# THE EFFECT OF ANTI-LYMPHOCYTIC ANTIBODY ON THE HUMORAL IMMUNE RESPONSES OF MICE

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To my wife

ANITA

who patiently put up with my extremely
irregular working hours during the course
of these studies

# ABSTRACT OF THESIS

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*Title of Thesis* The Effect of Anti-Lymphocytic Antibody on the Humoral Immune Responses of Mice

The effect of a single ALG preparation given on days -4 and -2 prior to antigen on the primary humoral immune responses of six mouse strains against different doses of sheep erythrocytes (SRBC), and type III pneumococcal polysaccharide (SSS-III) was tested. The effect of the same ALG preparation on the secondary humoral immune response of the same mouse strains was also examined, and in a limited number of experiments the antibody production in different immunoglobulin classes and subclasses was also measured. In addition, the effect of several other ALG preparations on the primary response against a standard dose of the above antigens as well as against bovine serum albumin (BSA) was also tested in selected mouse strains. These preparations were either obtained from a single horse at different stages of immunization or from different horses. In addition to the standard schedule the effect of an alternative protocol (day 0) on the immune response to SSS-III was also investigated in Balb/c mice. In a single experiment the anti-SSS-III response of ALG-treated CBA mice reconstituted with various lymphoid cells was also measured. Finally the immune responses of different mouse strains against SRBC, SSS-III and BSA were determined.

The results indicate that the ALG mainly used in the bulk of the studies suppressed the immune response of all six mouse strains against all the doses of the three antigens tested. It also suppressed the secondary responses of these strains to SRBC. This effect was also generally apparent in various immunoglobulin subclasses. However, in some cases, the response in certain immunoglobulin subclasses was enhanced or unaffected.

The other ALG preparations raised in different horses also suppressed the primary responses against SRBC and BSA, although their effect on anti-SSS-III response was variable. The results obtained with different ALG preparations obtained from the same horse at different stages of immunization suggest that IgG responses are generally more susceptible to ALG treatment than the IgM responses, and that ALG can suppress the thymus dependent responses more readily than the thymus independent ones.

The results of two ALG treatment schedules indicate that the ability of ALG to suppress at least anti-SSS-III response may largely depend on the time of its administration. Thus most of the ALG preparations were ineffective when given on the same day as SSS-III.

The response of ALG-treated and reconstituted mice (in one experiment) suggests that the impaired response of the ALG-treated mice to SSS-III may be partially restored by bone marrow, spleen or thymus cells.

The results of the measurement of humoral immune responses in different mouse strains indicate that significant inter-strain variation may exist in the magnitude of the primary immune responses to different antigens.

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The results of these studies have been discussed in the light of the present knowledge of the biological properties of anti-lymphocyte serum, the thymus dependence of different immune responses and the mechanism of immune reactions.

## PURPOSE OF THE STUDY

Detailed investigations undertaken in the last few years have unequivocally established the efficacy of anti-lymphocytic globulins as potent immunosuppressive agents. Their ability to suppress cell mediated immune responses has been well documented in a variety of experimental models (James, 1967a, 1969; Medawar, 1969; Sell, 1969; Lance, 1970c; Taub, 1970; Joost, 1970; Rolland and Nairn, 1972). However, their effect on humoral immune response has been less consistent, being influenced by various factors including the time of antigen administration, the nature of the antigen under test, the dose of the antigen, the incorporation of the antigen into adjuvants, and the strains of animals used. This inconsistency has given rise to conflicting views on the ability of anti-lymphocytic antibody to suppress humoral immune responses and has also led on occasions to the dangerous assumption that this reagent may not affect humoral immune responses.

In order to clarify this situation it was decided to determine the effect of anti-lymphocytic antibody on the humoral immune responses of a number of strains of mice against sheep erythrocytes, pneumococcal polysaccharide and bovine serum albumin.

## LIST OF TABLES

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a) From this work

1) GHAFFAR, A. and JAMES, K. (1973) 'The effect of antilymphocytic antibody in the humoral immune response of different strains of mice. II. The response of sheep erythrocytes.' Immunology, 24, 455.

2) GHAFFAR, A. and JAMES, K. (1973) 'The effect of antilymphocytic antibody in the humoral immune response of different strains of mice. III. The response to type III pneumococcus polysaccharide.' Immunology, in press.

3) GHAFFAR, A. and JAMES, K. (1973) 'The effect of antilymphocytic antibody on the humoral immune response of different strains of mice. IV. Variable effect of individual ALG preparations on the immune response to type III polysaccharide antigen.' Immunology, in press.

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1) FLAD, H., PLAYFAIR, J.H.L., GHAFFAR, A. and MISCHER, P.A. (1969) 'Strain differences in the liver autoantibody response of inbred mice.' Proc. Soc. exp. Biol. Med., 131, 121.

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# INTRODUCTION

# MECHANISM OF IMMUNE RESPONSES

The era of experimental immunology probably began with Jenner's classical experiments in 1798 in which he observed that the dairymaids who were exposed to cowpox virus were subsequently protected against smallpox virus infections. This line of approach to immunity pursued further by Pasteur and others ultimately formed the basis of the present vaccination practice for protection against infectious agents.

While by the end of the 19th century antibodies were believed to be the active agents for conferring immunity, a group of workers led by Metchnikoff were more impressed with the efficiency of certain white cells of blood and other body fluids to destroy bacteria. However, with the efforts of Wright and various other workers during the late 19th and very early 20th centuries, the two concepts were reconciled and it was accepted that although it was phagocytes which engulfed and destroyed bacteria, their activities were greatly assisted by the attachment of antibodies to the bacterial surface (Wright, 1906).

## Humoral immune response

The organs primarily involved in the establishment of immunity following infection or antigen administration

have long been suspected to be the spleen and lymph nodes. Concluding his lecture to the Harvey Society in 1907 Councilman clearly implied the association of the proliferative activity in lymphoid organs with the recovery from infections and subsequent immunity (Councilman, 1907). The sequence of events following immunization involving extensive hyperplasia in various lymphoid organs including the spleen, lymph nodes and the thymus have since been documented by various authors (Ehrich, 1929; Conway, 1938). Furthermore, McMaster and Hudack (1935) showed that the level of agglutinin extracted from the regional lymph node following intradermal injection of Salmonella was greater than that of non-regional node or serum. Their findings were later confirmed by Ehrich and Harris (1942) and De Gara and Angevine (1943).

The first popular candidate among the cells of lymphoid origin responsible for the production of antibody were macrophages but during the 1920's they were superceded in popularity by lymphocytes. However, the latter too lost ground following the observations of Bing and Plum (1937) who reported a positive correlation between hypergammaglobulinaemia and the increased plasmacytosis in the spleen. Furthermore, Undritz (1938) produced additional evidence on clinical grounds to connect plasma cells with antibody production.

In experimental animals the observations of Bjorneboe

and Gormsen (1943) and Fagraeus (1948a,b) also suggested an association between antibody production and plasma cells. This association was further strengthened following the development of radiolabelling and immunofluorescence techniques which permitted the direct demonstration of antibody on the cells of plasmacytic series (Coons, Leduc and Connolly, 1955; Ortega and Mellors, 1957; White, 1963).

Delayed hypersensitivity

Another type of immunological reaction, which could not be associated with any detectable circulating antibody nor with the development of plasma cells was first explored by Koch in the 1890's. The classical example of this type of immune response is the reaction of tuberculosis patients to tubercle antigen, but it includes contact dermatitis, sensitivity to chemicals and a number of other allergic reactions. This aspect of immunology did not receive much attention at the time but it is now accepted that delayed hypersensitivity, which this reaction was later termed, is an important part of the defence mechanism and can be induced by a variety of antigens.

In the 1930's the phenomenon of delayed hypersensitivity was extensively studied by Landsteiner and his colleagues. They showed that contact sensitivity

to picryl chloride could be transferred from sensitized to normal guinea pigs by means of peritoneal exudate cells (Landsteiner and Chase, 1942). Later it was shown that sensitivity to tuberculin could also be passively transferred in a similar way (Chase, 1945), although earlier attempts to transfer the reaction to non-sensitized animals by means of serum from sensitized animals had been unsuccessful (Zinsser and Mueller, 1925; Freund, 1926). It was also shown that passive transfer of the tuberculin reaction could be brought about by spleen or lymph node cells as well as by the peritoneal cells from a sensitized animal. The passive transfer of delayed hypersensitivity by means of lymphoid cells has now been confirmed many times (for example, Kirchheimer and Weiser, 1947; Stavitsky, 1948; Metaxas and Metaxas-Buhler, 1955; Turk, 1961).

Homograft immunity

It has long been known by surgeons that skin and tissue grafts from one individual to another are not normally accepted. The precise mechanism of rejection and the factors influencing it are not yet very clearly understood. There have been, however, a number of studies of the morphological changes both within the graft itself and within the regional lymph nodes.

Most of the early studies in transplantation were

limited to the transfer of tumours in rodents. The observations on the histology of regressing or rejected tumour grafts indicated the implication of the lymphoid tissue in the process of graft rejection (Jensen, 1903; Ehrlich, 1906; Bashford, Murray and Cramer, 1908; Levin, 1910; Da Fanco, 1912). In a very comprehensive report published in a monograph by Murphy in 1926, he emphasised the role lymphocytes played in the rejection of tumour grafts.

Fleisher (1917) implanted guinea pig kidney tissue subcutaneously in sensitized rabbits and found that the grafts soon underwent necrosis and the renal tissue became heavily infiltrated with lymphocytes and polymorphonuclear leucocytes. Similarly lymphocytic infiltration was observed by Loeb (1918) when he grafted thyroid tissue between guinea pigs.

The concept of genetically determined antigenic factors responsible for the immunological reactions in the host which eventually resulted in the rejection of histoincompatible tissue was first reported by Gorer in 1938. This was later expanded by Medawar in the 1940's (Medawar, 1944, 1945, 1946a,b) and marked the beginning of modern understanding of homograft reactions.

Mitchison (1953, 1954, 1955) demonstrated that transplantation immunity to certain tumours could be transferred to non-immune mice by injection with cells

from lymph nodes of tumour grafted mice. Billingham and his colleagues confirmed these results (Billingham, Brent and Medawar, 1954).

Role of the thymus in the immune response

The experiments reported by Miller (1961) showing that neonatal thymectomy in mice prevented the proper development of all cell mediated and some antibody responses could perhaps be claimed to mark the modern era of cellular immunology. An organ which hitherto seemed to have no significant function, whose removal in adult life had no apparently adverse consequences, acquired a unique importance. Its removal shortly following birth hampered the development of an entire population of lymphocytes including those in the periarteriolar regions of spleen and paracortical areas of lymph node, the majority of those in the thoracic duct lymph and about half of the peripheral blood population (Miller, 1962b; Parrott and De Sousa, 1966b). Following the initial studies of Miller (1962b) and Parrott and East (1962), it was soon recognized that neonatal thymectomy impaired the immune response against a variety of antigens. For example it resulted in a reduced antibody response to heterologous blood cells (Friedman, 1965; Sinclair, 1965; Law, 1966; Taylor and Wortis, 1968; Lemel, Cooper and Good, 1971; Dresser, 1972), heterologous serum proteins

(Good, Dalmasso, Martinez, Archer, Pierce and Papermaster, 1962; Jankovic, Waksman and Arnason, 1962; Arnason, De Vaux and Shaffner, 1964), bacterial antigens (Miller, Marshall and White, 1962; Arnason et al., 1964; Hadley and Elbers, 1964), viruses (East, Parrott, Chesterman and Pomerance, 1963; Foldes, Szeri, Banos, Anderlik and Balazs, 1964, 1965; Allison and Taylor, 1967; Mori, Tasaki, Kimura and Takeya, 1967; Mori, Kimoto and Takeya, 1970; Cheville, 1970) and various other antigens (Fahey, Barth and Law, 1965; Kruger and Gershon, 1971). Further studies also indicated that neonatal thymectomy alone or adult thymectomy combined with radiation also impaired the rejection of homografts (Miller, 1962a,b; Dalmasso, Martinez and Good, 1962; Davis, Tyan and Cole, 1964; Sosin, Hilgard and Martinez, 1966; Weston, Carter, Leuchars, Wallis and Davies, 1972) and the development of delayed type hypersensitivity reactions to a variety of agents (Arnason, Jankovic, Waksman and Wennersten, 1962; De Sousa and Parrott, 1969).

Since the development of techniques for selectively eliminating 'T' cells (Reif and Allen, 1964, 1966; Boyse, Miyazawa, Aoki and Old, 1968; Raff, 1969; Schlesinger and Yron, 1969; Aoki, Hammerling, deHarven, Boyse and Old, 1969), and 'B' cells (Raff, Nase and Mitchison, 1971; Basten, Sprent and Miller, 1971; Miller, Sprent, Basten and Warner, 1972) it has been possible to further

establish the role of 'T' cells in antibody production (Greaves and Moller, 1970; Raff, 1970; Schlesinger, 1970; Mitchison, 1971b; Miller and Sprent, 1971) and homograft rejection and other cell mediated immune responses (Miller, Brunner, Sprent, Russell and Mitchell, 1971; Cantor, 1972; Sprent and Miller, 1972; Moller, 1971; Rouse, Rölinghoff and Warner, 1972).

Cell co-operation in immunity

From the findings of Miller and Good and his colleagues (Good, Kelly, Rotstein and Varco, 1962) in mammals and those of Warner and Szenberg (1964) in the chicken it became apparent that the lymphoid cell populations could be divided into at least two compartments - one which required to be processed by the thymus to gain immunocompetence and the other which did not require the influence of thymus and which derived its origin from bursa in the chicken or bone marrow in the mammal. The former are popularly known as 'T' cells while the latter have been designated 'T-independent' or 'B' cells (Roitt, Greaves, Torrigiani, Brostoff and Playfair, 1969).

Using irradiated mice which were subsequently injected with cells of thymic or bone marrow origin or both, together with sheep erythrocytes, Claman and his colleagues showed that the number of antibody producing cells found in the spleens of animals repopulated with thymus and bone

marrow cells were far greater than the sum of those obtained when thymus or bone marrow cells alone were given (Claman, Chaperon and Triplet, 1966). This increase in immunological response due to the synergistic effect of the two cell populations is now commonly known as thymus-bone marrow co-operation. Other examples of similar synergistic co-operative effects have been demonstrated in immune responses against BSA (Taylor, 1969) and against haptens in conjunction with carriers (Rajewsky, Schirrmacher, Nase and Jerne, 1969; Mitchison, 1971a; Taylor and Iverson, 1971). Furthermore, experimental results have been reported showing similar co-operative effects between thymic and marrow cells in the cell mediated immune systems (Argyris, 1968; Hilgard, 1970; Barchilon and Gershon, 1970; Trident, Biasi, Chieco-Bianchi and Fiore-Donati, 1971).

Thymus dependence of immune responses

In the preceding sections it has been suggested that the presence of thymus derived cells are essential to initiate a humoral immune response to a variety of antigens. Nevertheless, there are a variety of antigens which do not require the participation of 'T' cells to initiate an immune response (Humphrey, Parrott and East, 1964; Parrott and De Sousa, 1966a; Davies, Carter, Leuchars, Wallis and Dietrich, 1969; Kerbel and Eidenger, 1971;

Feldman and Basten, 1971; Andersson and Blomgren, 1971). Moreover, in the case of the so called thymus-dependent antigens, it has been reported that an increase in antigen dose may overcome the need for the helper effect of thymus derived cells (Sinclair and Elliott, 1968; Taylor and Wortis, 1968; Tyan, Herzenberg and Gibbs, 1969; Playfair and Purves, 1971; Haskill, Marbrook and Elliott, 1971).

Nature of antibody producing cells

Although the presence of thymus, or cells of thymic origin, are essential for the production of antibody against a number of antigens, the production of the antibody itself is not physically carried out by these cells (Davies, Leuchars, Wallis, Merchant and Elliott, 1967). It would appear that the function of antibody production is carried out by the bone marrow derived population of plasma cells (Klein and Herzenburgh, 1967; Mitchell and Miller, 1968; Nossal, Cunningham, Mitchell and Miller, 1968).

Role of macrophages

It has long been known that a large part of injected antigen is taken up by macrophages, although the significance of this uptake in relation to the initiation of immune response has been the point of considerable debate.

However, the studies of Gallily and Feldman in irradiated animals indicated that the presence of a macrophage rich population of peritoneal exudate cells was essential to initiate an immune response in the irradiated recipient of various lymphoid cells (Gallily and Feldman, 1966, 1967). Similar observations supporting the importance of macrophages in initiating an antibody response have been reported by other workers using both in vivo and in vitro experimental models (Mosier, 1967; Hoffman, 1970; Sabet and Feldman, 1970; Feldman and Palmer, 1971; Tan and Gordon, 1971; Askov and Halliday, 1971). Furthermore, the requirement of macrophages has also been shown to be essential in various models of cell mediated immune responses (Hersh and Harris, 1968; Seeger and Oppenheim, 1970; Lonai and Feldman, 1971). However there are certain antigens which have been shown to initiate an immune response without the participation of macrophages (Shortman, Diener, Russell and Armstrong, 1970; Feldman and Palmer, 1971).

Stem cells and their origin

It has been known for some time that the lethal effects of radiation could be overcome by the administration of live haemopoietic cells from various organs. The principal role of the protective cell inoculum was found to be to restore granulocyte, and platelet formation,

although erythrocyte and lymphocyte production was also restored, and these cell types were traced to be of the donor bone marrow origin (Ford, Hamerton, Barnes and Loutit, 1956). It was also found that the best restorative cells were bone marrow cells, while spleen and peripheral blood cells were less effective and lymph node and thymus cells were without any effect.

Utilizing the spleen colony forming assay, it was shown that each colony arising following bone marrow injection into irradiated animals was the product of a single bone marrow cell and consisted of one type of cells, i.e. erythropoietic, granulocytic or megakaryocytic. However, if the cells from a single colony were subsequently injected into a second host, they gave rise to colonies of all three kinds. It was thus concluded that bone marrow contained a pluripotential lymphocyte-like stem cell (Cudkowicz, Bennet and Shearer, 1964).

By the injection of chromosomally marked cells from various sources and following their redistribution and localization, it was found that thymus, spleen and lymph node cells would settle in the spleen and lymph nodes, but not in the thymus. But, by contrast some bone marrow cells would colonize spleen, lymph nodes and thymus (Ford and Micklem, 1963; Micklem, Ford, Evans and Gray, 1966). Furthermore in extreme cases a single cell has been known to repopulate the entire lymphoid and haemopoietic tissue

of the body (Loutit, 1965). In summary, the bone marrow seems to be the ultimate source of stem cells in the adult animal, supplying the other haemopoietic and lymphoid organs.

In the preceding paragraphs, I have attempted to summarize only briefly the development of various aspects of immunology, and the present concept of cells involved in immune responses. I have restricted myself only to the aspects which are going to be essential for the understanding of the work described in the following chapters of this thesis. However, there are several recent reviews on the role of various cells and cell interactions in the immune response (Roitt, Greaves, Torrigiani, Brostoff and Playfair, 1969; Lischner and DiGeorge, 1969; Richter, 1970; Taylor and Iverson, 1970; Playfair, 1971; Miller, Basten, Sprent and Cheers, 1971).

## ANTI-LYMPHOCYTIC SERUM

### HISTORICAL

Once the relationship between the cells of the lymphoid system and immune response was established, it seemed reasonable to use antisera against such cells to suppress immune reactions. As early as 1899, when the role of various lymphoid cells in immunological responses was only in the speculative stage, Metchnikoff, who strongly believed that it was 'white' cells of the blood and other lymphoid tissues which were responsible for immunity against bacterial infections, raised antisera against rat or rabbit spleen and lymph node cells by immunizing guinea pigs with a saline emulsified preparation. These antisera showed leucoagglutinating and leucolytic activities with a degree of species specificity but not tissue specificity and reacted with polynuclear and mononuclear cells of rat as well as mast cells. He used these antisera to lower the resistance of its recipients to infections (Metchnikoff, 1899). These observations were followed by those of Besredka (1900) who showed that the lympholytic activities of rabbit anti-guinea pig lymph node sera were complement-dependent. He confirmed the species specificity described by Metchnikoff and, furthermore, indicated a small degree of tissue specificity. Two injections of his antisera into guinea pigs provoked

highly toxic effects with initial hypo- followed by hyper-leucocytosis. He also noted that anti-bone marrow serum was more effective than anti-mesenteric lymph node serum.

Funck (1900) raised antiserum against rabbit spleen and bone marrow cells in guinea pigs and found that although anti-spleen serum did not discriminate between mononuclear and polymorphonuclear cells of rabbit peritoneal exudate, anti-bone marrow serum destroyed polynuclear exudate cells more readily than mononuclear cells. Similar differences in the haemolytic activity of rabbit antisera against guinea pig lymph node and bone marrow cells were reported by Flexner (1902). While both anti-lymph node and anti-bone marrow sera produced hyperplasia of the spleen and lymph nodes in the recipient guinea pigs, only the former produced swelling and degeneration of lymph nodes. Bunting (1903) confirmed this finding by showing that goose anti-rabbit lymph node serum produced an absolute lymphopenia which lasted at least 4 days after injection, and that this was followed by marked lymphocytosis. He also reported a transient lymphopenia in recipients of normal goose serum, but this was not followed by lymphocytosis. The protracted lymphocytosis was not seen in the recipients of goose anti-bone marrow serum.

The first attempt to raise antiserum against thymus cells was made by Moorehead (1905) though this was unsuccessful. However, Ritchie (1908) produced duck

anti-guinea pig thymus serum, a single injection of which caused leucocytosis in the recipient guinea pigs.

Pappenheimer (1917) using the trypan blue dye exclusion test as criteria for the damage to cells *in vitro*, studied the effect of an anti-thymocyte serum on cells from thymus and those from lymph glands, and from the results showing an equal injury to the two types of cells, he concluded that these cells were histologically related. He also demonstrated that the lymphotoxic and lymphagglutinating factors in the antisera against both thymus and tonsillar lymphocytes were distinct from haemolytic and haemagglutinating factors since they could be separated by specific absorption.

Antisera against polymorphonuclear neutrophils were prepared by Ledingham and Bedson (1915) and Chew, Stephens and Lawrence (1936). These antisera exhibited some degree of specificity *in vitro* and when injected they caused a drop in the granulocyte but not the lymphocyte counts.

All these early efforts to raise antisera against lymphoid cells have yielded rather weak products with poor specificity. It was not until 1937 that Chew and Lawrence prepared a consistently powerful anti-guinea pig lymph node serum. Before use the antiserum was heat-inactivated to destroy the complement, absorbed with guinea pig erythrocytes to remove haemagglutinins and haemolysins, and with sheep erythrocytes to remove Forssman antibodies. A

single intraperitoneal injection of this serum into guinea pigs resulted in marked lymphopenia which lasted up to 40 hours and, a repeated course prolonged the effects for up to 10 days whereafter the lymphocyte counts returned towards the normal. These findings were confirmed by Cruickshank (1941). Using rabbit anti-rat lymphocyte serum, he demonstrated that the lymphopenia produced by this antiserum was limited to rats; it did not have the same effect in mice or rabbits.

Since the successful demonstration during the first half of this century of a definite role of lymphocytes in many immunological reactions it seemed reasonable to examine if antisera against lymphoid cells could alter the course of such reactions. Woodruff and his colleagues tried to influence antibody response against Salmonella antigen by administering anti-lymphocytic serum into previously immunized rats. Contrary to their expectations, this treatment failed to produce any significant changes in the antibody titres of the recipient animals (Woodruff, Foreman and Fraser, 1951). However, in these experiments they showed lymphocytotoxic activity of anti-lymphocytic serum in both <u>in vivo</u> (Woodruff, Foreman and Fraser, 1951) and <u>in vitro</u> systems (Woodruff and Foreman, 1951).

The first experiments showing a significant suppression of an immunological reaction by anti-lymphocytic antibody were reported by Inderbitzen (1956). He showed that a

single injection of rabbit anti-guinea pig lymphocyte serum suppressed contact sensitivity reaction against dinitro-chlorobenzene and tuberculin reaction in the recipient guinea pigs. By contrast, antiserum against neutrophils did not show any suppressive effect. His findings were confirmed by Wilhelm and his colleagues who suppressed contact sensitivity to dinitro-chlorobenzene in guinea pigs with rabbit anti-guinea pig lymphocyte serum (Wilhelm, Fisher and Cooke, 1958) and by Waksman, Arbouys and Arnason (1961). The latter group of workers also found that anti-lymphocytic serum caused a suppression of experimental autoallergic encephalomyelitis, but it had only a marginal effect on the reversed passive Arthus reaction and was without effect on the passive cutaneous anaphylaxis reaction. Woodruff and his colleagues, continuing their initial work with antiserum against rat thoracic duct lymphocytes, showed a significant prolongation of skin allografts by administering the antiserum prior to grafting (Woodruff and Anderson, 1963).

In the past decade numerous groups of workers have been investigating the effects and the mode of action of anti-lymphocytic sera using a variety of experimental models and an immense amount of data has accumulated showing the effects of anti-lymphocytic serum on different aspects of immunological reactions. The majority of these observations have been collectively summarized in several

recent reviews (James, 1967a, 1969; Medawar, 1969; Sell, 1969; Lance, 1970c; Taub, 1970; Jooste, 1970; Rolland and Nairn, 1972).

## PRODUCTION OF ANTI-LYMPHOCYTIC SERA

### Choice of animals

Antisera against lymphoid cells from experimental animals and man have been prepared in a variety of animals including rabbit, horse, sheep, cow, pig, monkey, goat, duck and guinea pig. Although the general body of evidence indicates that the choice of animal for the production of the antisera mainly depends on individual requirements and convenience, some of the observations suggest that the properties of anti-lymphocytic sera may be influenced by the species of animal they are produced in (cited by James, 1969, 1973a; Rolland and Nairn, 1972). On the basis of a relatively poor immunogenicity of rabbit globulin compared to equine globulin, the former species is favoured by some workers for the production of both human and murine anti-lymphocytic sera (Dresser and Gowland, 1964; Amemiya, Kashiwagi, Putnam and Starzl, 1970). Some workers also prefer the goat and the pig to the horse for raising anti-lymphocytic serum (Sutherland, Morriss and

Broad, 1970; Binns, Simpson, Nehlsen and Ruszkiewicz, 1970). In contrast Southworth and her colleagues (Southworth, Ohamian, Plate and Amos, 1970) have advocated the superiority of the horse over the goat and cow. Indeed the choice of animal species may also depend on the species of lymphoid cell (antigen) donor. It has been observed that while duck and chicken produce only weak and poor antisera against mouse lymphoid cells, they give a satisfactory antiserum when immunized against rabbit lymphoid cells (Reithmüller, 1967; Jooste, Lance, Levey, Medawar, Ruszkiewicz, Sharman and Taub, 1968). In conclusion it is not possible to lay any hard and fast rule on the most suitable species in which to raise anti-lymphocytic serum until the necessary information on certain relevant factors is available. These include the responsiveness of the antiserum producing species to vital lymphoid cell antigens, the distribution of antibody activity in immunoglobulin classes or subclasses and their biological functions, and the relative immunogenicity and tolerogenicity of immunoglobulin classes or subclasses in the recipient species.

Choice of antigen

Anti-lymphocytic sera with variable leucocytotoxic and immunosuppressive properties have been prepared by using cells from lymph nodes, peripheral blood, spleen, thoracic duct lymph and thymus. From various reports it

appears that antisera against thymus cells are more potent immunosuppressants than those against other lymphoid cells. Anti-spleen and anti-lymph node sera have been found to be poorer in their immunosuppressive properties and worse for their general toxicity (Nagaya and Sieker, 1965; Balner and Dersjant, 1967; Greaves, Tursi, Playfair, Torrigiani, Zamir, and Roitt, 1969; Wood and Vriesendorp, 1969; Argyris and Plotkin, 1970; Girardet, Glass, Patti and Gardner, 1971).

Anti-lymphocytic serum for experimental and therapeutic usage in man has been prepared against human thoracic duct and peripheral blood lymphocytes (Monaco, Wood and Russell, 1967; Starzl, Marchioro, Hutchison, Porter, Cerilli and Brettschneider, 1967; Traeger, Carraz, Fries, Perrin, Saubier, Bernhardt, Revillard, Bennett, Archimbaud and Brochier, 1969), infant thymic lymphocytes (Woodruff, 1967), and spleen cells (Iwasaki, Porter, Amend, Marchioro, Zuhlke and Starzl, 1967). Indeed certain authors have suggested the use of thoracic duct lymphocytes to raise antisera of better specificity and greater potency (Pichlmayr, 1970; Traeger, Fries, Revillard, Durix, Carraz and Plan, 1970).

In addition to intact lymphoid cells, lyophilized thymus cells (Jankovic, Isakovic and Petrovic, 1970), subcellular fractions of lymphocytes (Lance, Ford and Ruszkiewicz, 1968; Gozzo, Rule and Gentile, 1969; Warnatz, Schieffarth and Baier, 1969; Zola, Mosedale and

Thomas, 1970) and soluble extracts from mouse and human lymphocytes (Zola, Thomas, Mosedale and Courtenay, 1971) have been used.

Immunization schedule

In general, effective anti-lymphocyte sera are produced by two to four injections of lymphoid cell antigens given one or two weeks apart, subcutaneously or intravenously (Levey and Medawar, 1966a; Sutthiwan, Shorter, Hallenbeck and Elveback, 1969). The incorporation of adjuvant into the antigen preparation can result in the production of sera with higher antibody titres, although it is claimed that a lot of activity against non-lymphoid antigens may develop in the serum and consequently the product may give rise to considerable toxic reactions in the recipient animals (Jooste, Lance, Levey, Medawar, Ruszkiewicz, Sharman and Taub, 1968). In contrast, there is some suggestion that the adjuvant-produced antisera may on occasions be less toxic (Wood and Vriesendorp, 1969; Perper, Lyster, Monovich and Bowersox, 1970; Halbfass, Paravicini, Schaffer, Michaelis and Staib, 1971). However, the benefits of using adjuvants to raise ALS of greater immunosuppressive activity have often been found doubtful (Wood and Vriesendorp, 1969; Sterling, Elveback and Shorter, 1970).

## Active fractions

The immunosuppressive activity of ALS is associated with the γ-globulin fraction and the activity against lymphoid cells can be specifically absorbed by various lymphoid cells. The distribution of antibody activity in different immunoglobulin classes and subclasses may depend upon many factors known to influence the immune responses. However, the bulk of immunosuppressive activity in ALS is generally associated with the 7S γ-globulin fraction. This fraction is also rich in lymphoagglutinating, lymphocytotoxic and lymphocyte-transforming activities (James and Medawar, 1967; Woodruff, James, Anderson and Reid, 1967; James, 1969; Allardyce, Anderson, Vaerman and James, 1973).

# BIOLOGICAL PROPERTIES OF ANTI-LYMPHOCYTIC SERUM

## Effect on circulating lymphocytes

A single injection of anti-lymphocyte serum causes an acute fall in the circulating lymphocyte count (Chew and Lawrence, 1937; Woodruff and Anderson, 1963; Jeejeebhoy, 1965b; Gray, Monaco, Wood and Russell, 1966) which recovers fairly quickly, often despite the continuation of the anti-lymphocyte serum treatment (Nagaya and Sieker,

1965; Anderson, James and Woodruff, 1967). However, several authors have found that lymphopenia could be maintained for longer periods by frequent and prolonged administration of the anti-lymphocytic serum (Monaco, Abbott, Otherson, Simmons, Wood, Flax and Russell, 1966; Atai and Kelly, 1967; Pichlmayr, Brendel and Zenker, 1967; Clunie, Nolan, James, Watt and Woodruff, 1968; Denman and Frenkel, 1968b).

No permanent effects of chronic anti-lymphocyte serum treatment on other blood cell counts have been established. A transient reduction in granulocyte and monocyte counts has been reported but they return to normal more quickly than lymphocytes (Pichlmayr, Brendel and Zenker, 1967; Ono, Bell, Kashiwagi and Sterzl, 1969). Increased granulocyte counts have also been reported following anti-lymphocyte serum treatment (Sacks, Filippone and Hume, 1964; Ono, DeWitt, Wallace and Lindsey, 1969).

The significance of lymphopenia as an indication of immunosuppressive potency of anti-lymphocytic serum has been rather doubtful. Indeed correlations have been reported firstly between the degree of lymphopenia and graft survival by a particular anti-lymphocyte serum (Rule and Judd, 1968; Taub, 1969) and secondly between the return of lymphocyte counts to normal levels and the onset of graft rejection (Pichlmayr, Brendel and Zenker, 1967; Lawson, Ellis, Kirchheim and Hodges, 1967; Clunie, Nolan, James,

Watt and Woodruff, 1968). However, contrasting observations have been reported indicating a lack of correlation between lymphopenia and immunosuppression. For example, an anti-lymphocytic serum may produce lymphopenia but cause no significant prolongation of graft survival (Herman and Schloerb, 1967). Furthermore, a reverse relationship has also been observed (Grogan and Hardy, 1967; Guttman, Carpenter, Lindquist and Merrill, 1967; Simmons, Ozerkis and Hoehn, 1968).

Histological changes

The histological changes following treatment with anti-lymphocytic serum in the lymphoid organs are due to two factors; the destruction or removal of certain populations of lymphocytes, and the proliferation of other lymphocytes, plasma cells and reticulocytes. The secondary effects like increased phagocytosis and migration of lymphoid cells from primary lymphoid organs to the periphery may also contribute to the histological changes. Following anti-lymphocytic serum treatment, a marked reduction in the cortical region of the thymus has been noticed by several authors (Nagaya and Sieker, 1966a; Denman and Frenkel, 1967; Bitterman and Shorter, 1968; Levey and Taub, 1969; Simpson and Nehlsen, 1971). The thymic lymphopenia has been attributed by Denman and Frenkel (1968b) to the migration of small lymphocytes into the circulation rather

than due to a direct effect of anti-lymphocytic serum on the thymus itself. This is consistent with the electron microscopic observations made by Denman and Frenkel (1968a) who failed to find any damaged lymphocytes within the thymus.

Animals treated with anti-lymphocytic serum show a marked lymphocytic depletion in the periarteriolar areas of splenic white pulp with a reduction in the size and the number of lymph follicles (Nagaya and Sieker, 1966a; Denman and Frenkel, 1967; Taub and Lance, 1968; Simpson and Nehlsen, 1971). Local areas of necrosis and signs of local destruction of lymphocytes have been noticed by both light (Gray, Monaco, Wood and Russell, 1966) and electron microscopy (Denman and Frenkel, 1968b). Concurrent hyperplasia of the red pulp of the spleen is also associated with the anti-lymphocytic serum treatment but this varies with different anti-lymphocytic serum preparations. An increase in reticulocytes, plasma cells and their precursors and large pyroninophilic cell populations has frequently been reported (Taub and Lance, 1968; Barth, Hunter, Southworth and Rabson, 1969; Simpson and Nehlsen, 1971).

Marked depletion of lymphocytes in the paracortical areas of lymph nodes has been noticed following a single dose of anti-lymphocytic serum. This is associated with damage to and disintegration of lymphocytes in these areas (Woodruff and Anderson, 1963; Turk and Willoughby, 1967; Denman and Frenkel, 1968a; Taub, 1969). A prolonged

course of anti-lymphocyte serum may also result in the necrosis of these areas (Gray, Monaco, Wood and Russell, 1966). Several investigators have noted hyperplasia of plasma cells, reticulocytes and their precursors in the lymph nodes of anti-lymphocytic serum treated mice (Gray, Monaco, Wood and Russell, 1966; Levey and Medawar, 1966a; Balner and Dersjant, 1967; Taub and Lance, 1968).

The hyperplasia, both in the spleen and the lymph nodes has been attributed to the immune response of the recipient animals to constituents of the anti-lymphocytic serum since similar although less rigorous changes occur after the administration of normal rabbit serum (Taub and Lance, 1968; Simpson and Nehlsen, 1971). In animals previously made tolerant to normal serum IgG, anti-lymphocytic serum IgG produced considerably less hyperplasia (Taub and Lance, 1968; Rodriguez-Paradisi, Thierfelder, Götze, Eulitz and Beil, 1970).

In vivo localization of anti-lymphocytic serum

There is no general agreement on the quantitative distribution of anti-lymphocytic globulin in various lymphoid organs. This has probably arisen from differing sensitivities of the methods used for localizing and the very low levels of the activity being measured. There is however, a substantial amount of evidence that anti-lymphocytic antibodies combine mainly with peripheral lymphocytes and

only small amounts find access to the central lymphoid organs (reviewed by Rolland and Nairn, 1972).

Effect on tissue grafts

The first attempts to prolong skin allografts using anti-lymphocytic serum were made by Waksman and his colleagues in guinea pigs (Waksman, Arbuoys and Arnason, 1961). In these experiments they achieved only a marginal prolongation of skin allografts. The first really significant prolongation of skin allografts by anti-lymphocytic serum was demonstrated by Woodruff and Anderson (1963). Various workers have since confirmed the prolongation of skin allografts in different species including rats (Woodruff and Anderson, 1964; Nagaya and Sieker, 1965; Jeejeebhoy, 1965a,b, 1967), mice (Levey and Medawar, 1966a,b; Monaco, Wood, Gray and Russell, 1966), pigs (Lucke, Immelman, Symes and Hunt, 1968), monkeys (Balner and Dersjant, 1967) and man (Monaco, Wood and Russell, 1967; Monaco, Wood, van der Werf and Russell, 1967; Najarian, Simmons, Moberg, Gewurz, Soll and Tallent, 1970).

The immunosuppressive effects of anti-lymphocytic sera are not confined to skin allograft rejections. They have been found effective in combating the rejection of renal allografts in various species (Abaza, Nolan, Watt and Woodruff, 1966; Monaco, Abbott, Otherson, Simmons, Wood, Flax and Russell, 1966; Lawson, Ellis and Hodges,

1966; Huntley, Taylor, Iwasaki, Marchioro, Jeejeebhoy, Porter and Starzl, 1966; Pichlmayr, 1967; Starzl, Marchioro, Porter, Iwasaki and Cerrilli, 1967; Starzl, Groth, Terasaki, Putnam, Brettschneider and Marchioro, 1968), homologous cardiac grafts in dogs (Halpern, Cachera, Lacombe, Hathaway, Crepin, Huang, Leandri, Laurent and Dubost, 1968), homologous liver transplants in the dog and man (Starzl, Marchioro, Faris, McCardle and Iwasaki, 1966) and corneal allografts in rabbits (Waltman, Faulkner and Burde, 1969). Anti-lymphocytic sera have been effective in prolonging not only allogeneic tissue, but they have also been shown to facilitate the survival of heterografts of skin (Monaco, Wood and Russell, 1966), human mammary tissue (Lance and Medawar, 1968) and human neoplastic tissue (Phillips and Gazet, 1967) in mice and rats.

The immunosuppressive effects of anti-lymphocyte sera on homograft survival have been shown to be affected by the dose of the antiserum (Levey and Medawar, 1966b; Lance, 1968; Symes, Immelman, Lucke and Mansell, 1969; Najarian, Simmons, Moberg, Gewurz, Soll and Tallent, 1970), timing of anti-lymphocyte serum treatment in relation to the application of graft (Monaco, Wood, Gray and Russell, 1966; Starzl, Marchioro, Hutchison, Porter, Cerrilli and Brettschneider, 1967; Clunie, Nolan, James, Watt and Woodruff, 1968; Grogan, Moynihan and Hardy, 1968; Floersheim, 1969; Van Bekkum, Heystek and Marquet, 1969)

and the prior sensitization of the recipient (Levey and Medawar, 1966b), but not by the antigenic disparity between the recipient and the donor strains (Levey and Medawar, 1966a), although it may be affected by interspecies differences (Monaco, Wood and Russell, 1966).

Effect on other cell mediated immune reactions

Apart from their effect on the survival of skin and tissue allografts, anti-lymphocytic sera have also been shown to influence various other cell mediated immunological phenomena. Anti-lymphocyte serum treatment of either the donor or the recipient of semi-allogeneic spleen cells suppresses the graft versus host disease in mice (Boak, Fox and Wilson, 1967; Brent, Courtenay and Gowland, 1967; Naysmith and James, 1968; Boak, Dagher, Carson and Wilson, 1968; Mandel and Asofsky, 1968; Floersheim and Ruskiewicz, 1969). Furthermore, anti-lymphocyte serum is capable of abolishing delayed hypersensitivity reactions to tuberculin in guinea pigs, mice, rats and humans (Inderbitzen, 1956; Waksman, Arbuoys and Arnason, 1961; Russe and Crowle, 1965; Currey and Ziff, 1967; Brunstetter and Claman, 1968), to diphtheria toxoid in guinea pigs (Waksman, Arbuoys and Arnason, 1961), to bovine serum albumin in rats (Nagaya and Sieker, 1966b), to ovalbumin in mice (Russe and Crowle, 1965) and to microbial antigens in mice and humans (Monaco, Wood and Russell, 1967; Brunstetter and Claman, 1968;

Domingo and Warren, 1968; Adamson and Cozad, 1969). Contact sensitivity reactions to dinitro-chlorobenzene have also been suppressed by anti-lymphocyte serum in guinea pigs and chimpanzees (Inderbitzin, 1956; Wilhelm, Fisher and Cooke, 1958; Waksman, Arbuoys and Arnason, 1961; Turk and Willoughby, 1967; Balner, Eysvoogel and Cleton, 1968).

These findings provide formidable evidence of an effect of anti-lymphocyte sera on those lymphoid cells and tissues which are associated with the development of cell mediated immune reactions in various species. Other suppressive effects of anti-lymphocytic sera like those in immediate hypersensitivity (Waksman, Arbuoys and Arnason, 1961; Polak and Turk, 1969), Arthus-type reactions to ovalbumin (Turk and Polak, 1969) and other acute inflammatory reactions to various agents (Waksman, Arbuoys and Arnason, 1961; Morris, Bondoc and Burke, 1966; Turk, Willoughby and Stevens, 1968; Perper, Glen and Monovich, 1969) may be due to the reactivity of antisera against factors other than lymphocytes. Some of these activities have been partly attributed to antibodies against lymph node permeability factor (Turk and Willoughby, 1967), macrophage components (Field and Hughes, 1969) or serum complement (Willoughby, Polak and Turk, 1968; Turk and Willoughby, 1969) or to changes in local histamine concentrations (Inderbitzin, 1956; Wolf-Jurgensen and Zachariae, 1970).

## THE EFFECT OF ANTI-LYMPHOCYTE SERUM ON THE HUMORAL IMMUNE RESPONSE

Whereas there is a general agreement over the effectiveness of anti-lymphocytic sera in prolonging the survival of homologous and heterologous grafts and in suppressing various other immunological reactions in which cell mediated immune mechanisms are chiefly implicated, the views relating to their effect on humoral immune reactions are far less uniform. Although primary immune responses to a number of antigens have been shown to be impaired in anti-lymphocyte serum treated animals, there are certain antigens which appear refractive to the reagent. Furthermore, rather divergent views are held regarding the effect of anti-lymphocyte serum on the secondary humoral immune responses to a variety of antigens.

Certain results reported by some workers indicate a clear dichotomy in the effectiveness of anti-lymphocytic serum to suppress humoral and cellular immune responses. For example, Lance and Batchelor (1968) showed that CBA mice chronically treated with anti-lymphocyte serum and grafted with A-strain skin ultimately developed circulating antibodies directed against A-strain histocompatibility antigens, although at this time they still showed a complete suppression of transplantation immunity to these antigens.

Another anomaly was observed in animals which were

treated with anti-lymphocyte serum for a significant length of time prior to the application of allografts. These animals developed antibodies to antilymphocytic globulin although their response to allografts was moderately to severely impaired (Currey and Ziff, 1966; Guttman, Carpenter, Lindquist and Merrill, 1967; Anderson, Clark, James, Reid and Woodruff, 1967; Jasin, Lourie, Currey and Ziff, 1968). These antibodies are made in increasing amounts as anti-lymphocyte treatment is continued despite the severe suppression of various cell mediated immunological reactions. These observations on the production of antibody to anti-lymphocyte globulin have been attributed to relatively greater immunogenicity of anti-lymphocyte globulin in relation to its normal globulin counterpart (Lance and Dresser, 1967; Howard, Asfis and Woodruff, 1968; Howard, Dougherty and Mergenhagen, 1968; Golub and Weigle, 1969; Kind and Ako, 1971; Revillard and Brochier, 1971). Contrary to these findings, others have claimed the anti-lymphocyte serum exerts an antidotal effect (Monaco, Wood, Gray and Russell, 1966; Iwasaki, Porter, Amend, Marchioro, Zuhlke and Starzl, 1967; Rule and Judd, 1968; Levey and Medawar, 1966a,b).

These discrepancies led to the belief that anti-lymphocyte serum could suppress cell mediated immune responses without affecting the humoral immunity. However, the observations made by other workers with heterologous antigens proved contrary to this concept.

The effects of anti-lymphocyte serum on humoral immune responses to a variety of antigens have been studied and the results are far from uniform.

Effect on the primary immune response

The effect of anti-lymphocyte serum has been most extensively studied in mice challenged with sheep erythrocytes. The earlier attempts to influence the humoral antibody responses with anti-lymphocyte serum were rather inconsistent. For instance, Jeejeebhoy (1965a) was unable to suppress by anti-lymphocyte serum treatment the antibody response in rats against sheep erythrocytes and tetanus toxoid. Similarly, Monaco and his colleagues found that anti-lymphocyte serum had very little effect on the antibody response of mice to sheep erythrocytes (Monaco, Wood and Russell, 1965). Nevertheless, anti-lymphocyte serum given to rats and mice following adult thymectomy had a significant suppressive effect on the antibody response to these antigens (Jeejeebhoy, 1965a; Monaco, Wood and Russell, 1965; Leuchars, Wallis and Davies, 1968).

In contrast the later studies showed that anti-lymphocyte serum could suppress the primary immune response to various antigens. Monaco and his colleagues reported a successful suppression of primary antibody response to sheep erythrocytes in mice treated with anti-lymphocyte serum (Monaco, Wood, Gray and Russell, 1966). Similarly,

successful suppression of primary anti-sheep erythrocyte response has been reported by several authors in both rats and mice (James and Anderson, 1967; Frenkel, Lieberman and Baum, 1967; Leuchars, Wallis and Davies, 1968; James, Pullar and James, 1968; Riethmuller, Stein and Hausen, 1968; Witz, Yagi and Pressman, 1968; Stewart and Cohen, 1969; Baum, Lieberman and Frenkel, 1969; Barth, Hunter and Southworth, 1969).

Apart from sheep erythrocytes, the primary immune responses to other antigens have been shown to be suppressed by anti-lymphocyte serum treatment. For example, anti-lymphocytic serum has been shown to suppress primary immune responses to bovine serum albumin in rats (James and Jubb, 1967; James, 1968, 1970) and in mice (James and Milne, 1971, 1972; Marshall and Knight, 1969; Mitchison, 1970, 1971b; Lance, 1970a,b; Nehlsen, 1971a,b) Salmonella-II antigen (Gray, Monaco and Russell, 1964; Lance, 1970b), E. coli lipopolysaccharide (Allen, Friedman and Mills, 1969), keyhole limpet haemocyanin (Nehlsen, 1971a,b), goose erythrocytes and horse spleen ferritin (Kerbel and Eidinger, 1971) and type III pneumococcal polysaccharide (James and Milne, 1971). In addition anti-lymphocyte serum has been shown to suppress complement fixing antibody response to lymphocytic choriomeningitis virus (Lundstedt and Volkart, 1967; Volkart and Lundstedt, 1968) and neutralizing antibody response against mouse pox virus (Blanden, 1970).

Anti-lymphocyte sera not only suppress antibody responses to heterologous antigens, they have been shown to exert their immunosuppressive effects in experimental as well as spontaneous autoimmune reactions. Suppression of clinical manifestations has been achieved in cases of adjuvant arthritis in rats (Currey and Ziff, 1967; 1968; Possanza and Stewart, 1970), allergic encephalomyelitis in guinea pigs (Waksman, Arbuoys and Arnason, 1961; Liebowitz, Lessof and Kennedy, 1968; Field, 1969), thyroiditis in rats (Kalden, James, Williamson and Irvine, 1968) and nephritis in rats (Barabas, James and Lannigan, 1969). Furthermore Denman and his colleagues have reported an impaired development of haemolytic anaemia with positive anti-globulin reaction in New Zealand Black mice treated with anti-lymphocyte serum (Denman, Denman and Holborow, 1967; Denman, Russell, Loewi and Denman, 1971).

Despite the bulk of evidence on the suppressive effect of anti-lymphocyte serum on the humoral immune response, there are a number of reports indicating the inability of this reagent to suppress antibody formation against certain antigens. Whereas Nehlsen (1971a,b) showed suppression of antibody response to keyhole limpet haemocyanin in CBA mice, Kerbel and Eidinger (1971) failed to achieve this effect in Swiss mice in the earlier phase of the immune response to this antigen. Similarly, anti-lymphocyte antibody has often failed to suppress antibody responses

to rat erythrocytes, polyoma virus (Nehlsen, 1971a), Brucella abortus (Pichlmayr, Brendel and Zenker, 1967) and Salmonella typhi 'H' antigen (Lance, 1968). The reason for this discrepancy may lie in the design of various experimental models.

In addition several reports have indicated that the immune responses which require little or no participation by thymus cells are augmented by the anti-lymphocyte serum treatment. For example anti-lymphocyte serum treatment resulted in an enhanced 19S as well as 7S antibody response to keyhole limpet haemocyanin in rats (Frenkel, Lieberman and Baum, 1967; Baum, Lieberman and Frenkel, 1969). Similar findings were reported by Kerbel and Eidinger (1971) who noted an enhancement of the antibody response to this antigen in anti-lymphocyte serum treated mice. Furthermore, Baker and his colleagues have observed that anti-lymphocyte serum pretreatment caused up to 10-fold increase in the antibody plaque forming cell response in Balb/c mice immunized with an optimal dose of type III pneumococcal polysaccharide antigen (Baker, Barth, Stashak and Amsbaugh, 1970; Baker, Stashak, Amsbaugh, Prescot and Barth, 1970; Barth, Baker, Stashak and Amsbaugh, 1971). The latter group of workers attributed this enhancement to the preferential inactivation of a suppressed or 'T' cell population which controlled or inhibited antibody formation by 'B' cells.

Effect on the secondary immune response

Reports on the efficacy of anti-lymphocyte serum to influence secondary humoral immune responses are far more controversial than those on the primary ones. Some workers have reported a suppression by their antisera of the secondary response to certain antigens while others have shown little or no effect of anti-lymphocyte serum on the secondary response to the same antigens. Also, the ability of an anti-lymphocytic serum to suppress secondary responses appears to be influenced by the antigen under test. Furthermore, the species and the strain of animals used in these studies seems to contribute towards this discrepancy.

Like the studies undertaken on the effect of anti-lymphocyte serum on the primary responses, the bulk of studies on the secondary humoral immune responses have also been carried out with sheep erythrocytes and bovine serum albumin. The earlier efforts to suppress the secondary antibody response to sheep erythrocytes proved unsuccessful (Monaco, Wood and Russell, 1965; Monaco, Wood, Gray and Russell, 1966), unless the animals were thymectomized in adult life prior to receiving the anti-lymphocyte serum treatment (Monaco, Wood and Russell, 1965). These findings were supported by the observations of James and Anderson (1967) and Baum, Lieberman and Frenkel (1969), who also failed to significantly suppress the secondary

haemagglutinin response to sheep erythrocytes in rats. Similarly experiments performed in rats (James and Jubb, 1967) and in CBA mice (Lance, 1968a, 1970a,b) indicated that anti-lymphocyte serum had very little immunosuppressive effect on the secondary humoral immune response to bovine serum albumin. However, there was a marginal suppression of the secondary humoral response to Salmonella 'H' antigen in CBA mice when anti-lymphocyte serum was administered prior to the priming of these mice (Lance, 1970a).

The most convincing evidence of the suppression of the secondary humoral immune response was found by Barth and Southworth (1968) who observed that anti-lymphocytic antibody treatment prior to either priming or the secondary challenge dose severely impaired both IgM and IgG responses of Balb/c mice, although the secondary agglutinin responses in these mice remained comparable in both anti-lymphocyte globulin treated and control animals. Similarly CBA mice treated with four doses of anti-lymphocyte serum on alternate days before the primary immunization, when given a secondary challenge with sheep erythrocytes five weeks later, showed significantly depressed haemagglutinin titres. The secondary response, just like the primary response, was more affected by anti-lymphocyte serum in thymectomized mice than in sham-operated ones (Leuchars, Wallis and Davies, 1968).

In contrast to the aforementioned findings with bovine serum albumin, Marshall and Knight (1969) observed a suppression of the secondary response to this antigen administered in Freund's complete adjuvant following anti-lymphocytic serum treatment. The suppression was directly proportional to the priming antigen dose. The fact that these authors measured the antibody response by passive haemagglutination technique may be of some significance.

## FACTORS INFLUENCING THE IMMUNOSUPPRESSIVE EFFECT OF ANTI-LYMPHOCYTE SERUM

### The dose of the anti-lymphocyte serum

One of the factors commonly accepted to have a bearing on the suppression of immune responses by anti-lymphocyte serum is the dose of the antiserum administered. This would inevitably be related to the properties of an individual anti-lymphocyte serum preparation: its specificity and potency. Thus graft prolongation has been shown to be directly related to the dose of anti-lymphocyte globulin administered (Bitterman and Shorter, 1968; Grogan, Moynihan and Hardy, 1968; Mandel and Asofsky, 1968; Symes, Immelman, Lucke and Mansell, 1969; Najarian, Simmons, Moberg, Gewurz, Soll and Tallent, 1970). It has also been shown that a

large amount is more effective when given as a number of smaller doses (Lance, 1968; Levey and Medawar, 1966b; Symes, Immelman, Lucke and Mansell, 1969; Wood and Vriesendorp, 1969). This relationship between the dose of antiserum and the degree of immunosuppression has been shown to hold true for humoral immune responses as well (Monaco, Wood, Gray and Russell, 1966; James and Milne, 1972).

Timing schedule of the anti-lymphocyte serum treatment

Although the time of anti-lymphocyte serum administration in relation to the application of grafts is not so critical for its prolongation (Levey and Medawar, 1966b; Greaves, Tursi, Playfair, Torrigiani, Zamir and Roitt, 1969; Jooste, Lance, Levey, Medawar, Ruszkiewicz, Sherman and Taub, 1968), this is very important in the suppression of humoral immune responses to a variety of antigens. Berenbaum (1967) showed that the immunosuppressive effect of the anti-lymphocyte serum was maximum when given 3 days before sheep erythrocytes, but it waned as the time between the antiserum and the antigen was reduced and it completely vanished when the antiserum was delayed up to 2-3 days after the sensitization. In parallel studies, James (1967b) noticed the inefficiency of anti-lymphocyte serum to suppress the immune response of rats to bovine serum albumin when administered after the antigen.

These findings were confirmed by Baroni and his colleagues who found that although anti-lymphocyte serum given up to 30 days before sheep erythrocytes effectively suppressed the immune response, it was completely ineffective when given two days after the antigen (Baroni, Kimball, Ward and Wagar, 1969). Similar observations have been recorded by various other workers in a variety of experimental models (Moller and Zukoski, 1968a,b; Barth, Southworth and Burger, 1968; Riethmuller, Riethmuller, Stein and Hausen, 1968; Kayibanda and Amiel, 1970; Lance, 1970a,b). In certain instances anti-lymphocyte serum has been shown to be ineffective when given as early as four hours after the antigen (Kalden, James, Williamson and Irvine, 1969).

The dose of immunizing antigen

The amount of antigen to which anti-lymphocyte serum can successfully suppress the immune response seems as important a factor as the amount of anti-lymphocyte serum administered. Lance (1970a,b) showed that while anti-lymphocyte serum impaired the immune response to small doses of bovine serum albumin in mice it had little, or no effect on the immune response to higher doses of this antigen. Similar observations were made by Argyris and Plotkins (1970) using sheep erythrocytes as antigen. Furthermore, James and Milne (1972) observed that the degree of suppression by anti-lymphocyte serum of anti-

bovine serum albumin response in mice was inversely related to the antigen dose administered.

Incorporation of adjuvants

Incorporation of the antigen into adjuvant has also been shown to diminish the immunosuppressive effect of anti-lymphocyte serum in rats (Allardyce, Hunter and James, 1970). These authors observed that anti-lymphocyte serum suppressed the primary immune response to bovine serum albumin administered as alum precipitate or in Freund's incomplete adjuvant, but did not affect the final response to the antigen if given in Freund's complete adjuvant. Furthermore, experiments undertaken in mice also suggest that the ability of anti-lymphocytic serum to suppress the response to alum-precipitated bovine serum albumin is often curtailed by the incorporation of Bordetella pertussis into the antigen (James and Milne, 1972).

Strain and species of animals under test

James and his colleagues showed that the ability of anti-lymphocyte serum to suppress humoral immune response in rats was influenced by the strain of animals under test (James, Pullar and James, 1969). Similar observations were made by James and Milne (1972) in mice. Their anti-lymphocytic serum preparation failed to suppress the primary immune response to low doses of bovine serum albumin in

Balb/c mice, whereas it was extremely effective in several other strains tested. This inability to suppress these immune responses was not related to the immune responsiveness of the strain of animals under test or the source of lymphoid antigen used in the production of the antiserum.

Apart from those listed above, there are several other factors which may influence the ability of the anti-lymphocyte serum to suppress immune responses. These factors may include the variation in the properties of different antisera although raised in the same manner (Jeejeebhoy and Singla, 1972a), differences in the antigen used to raise the anti-lymphocyte serum (Argyris and Plotkin, 1970), and also differences in the ability of the animal in which the anti-serum is raised to respond to the lymphoid antigens.

From the spectrum of observations listed, it is apparent that part of the controversy relating to the ability of anti-lymphocyte sera to suppress the humoral immune responses has undoubtedly arisen from the fact that most of the studies on this aspect of the immune response have been carried out in either one strain of animals or with a single standard dose of one particular antigen. Furthermore, in the majority of cases, either a single preparation of anti-lymphocytic serum has been used or the product has varied from one experiment to the other in the same laboratory.

It was therefore felt necessary to investigate the effect of this rather powerful immunosuppressive agent

(which is in some quarters still regarded as a selective inhibitor of cell mediated immunity: Lance and Batchelor, 1968; Lance, 1970a) on the primary and secondary humoral immune responses of a variety of strains of mice to varying doses of different antigens. The antigens were chosen on the basis of their thymus dependency, because it has been suggested that anti-lymphocyte sera act discriminately upon the cells of thymic origin (Martin and Miller, 1968; Medawar, 1969; Mitchison, 1971b).

In the bulk of the studies to be reported, a single preparation of the anti-lymphocyte globulin was used. However, to exclude the possibility of a single antiserum being an atypical product, several other preparations have been tested in selected experiments.

# MATERIALS AND METHODS

# MATERIALS AND METHODS

## MICE

Mice of the A/HeJ, C57Bl, Balb/c, DBA/1, CBA and C3H strains were used in these studies. Generally these mice were between two and three months old when used. The details of the origin, $H^2$ histocompatibility group and the source of these mice have been listed in Table 1. The A/HeJ, CBA and C3H mice were inbred in our own animal house at the Department of Surgery, Edinburgh University. The Balb/c and DBA/1 mice were kindly provided by Dr. J.G. Howard of the Wellcome Research Laboratories, Beckenham, Kent. These had been inbred in their animal house at Beckenham from the original breeding pairs obtained from the source listed in Table 1. The C57Bl mice were purchased from the Animal Suppliers Limited, Colindale, London N.W.11. The origin of their breeding pairs has also been indicated in Table 1. Throughout these studies only male mice were used.

Experimental mice were housed in plastic boxes with five to six mice in each box. The temperature of the mouse room was maintained at 22°C and a constant air exchange rate to provide adequate ventilation was always ensured. The mouse boxes were lined with wood shavings. Mice were fed on a commercial mouse cake diet (McGregors of Leith, Edinburgh). Water and food were available to

TABLE 1 - DETAILS OF MICE USED*

| STRAIN | H-2 TYPE | S O U R C E |
|---|---|---|
| A/HeJ | a | Jackson Laboratories, Bar Harbour. |
| $C_{57}Bl$ | b | Animal Supplier Limited, London. |
| Balb/c | d | Chester Beatty Research Laboratories, London. |
| DBA/1 | q | Chester Beatty Research Laboratories, London. |
| CBA | k | M.R.C. Laboratory Animal Centre, Carshalton, Surrey. |
| $C_3H$ | k | Radiobiological Research Unit, Harwell. |

*Mice generally 10-12 week old males

mice *ad libitum*.

## ANTI-LYMPHOCYTE SERUM

The anti-lymphocyte sera used in these studies were all raised in horses against mouse thymocytes. The batches 1a, 1b, 1c, and 1d were obtained from a single horse at various stages of immunization, with a pool of dispersed thymus cells from A/HeJ, CBA, C3H, and C57Bl mice. The anti-lymphocyte globulin preparations 2, 3, and 4 were obtained from antisera raised in different horses against mouse thymus cells. These were generously provided by the Wellcome Research Laboratories, Beckenham, Kent. Details of the immunization schedules employed in raising the different anti-lymphocyte sera have been listed in Table 2.

## Preparation of IgG from normal and anti-lymphocyte sera

The serum was inactivated by incubating it at 56°C for 30 minutes after which the pH of the serum was adjusted to 8.0 by the addition of N/1 sodium hydroxide. An equal volume of 28 per cent sodium sulphate (w/v) was then slowly added to the serum with continuous stirring; the final salt concentration reached was 14 per cent. The globulin precipitate was then recovered by centrifugation

TABLE 2 - A SUMMARY OF THE IMMUNIZATION SCHEDULES USED IN THE PRODUCTION OF THE VARIOUS HORSE ANTI-MOUSE LYMPHOCYTE PREPARATIONS

| Prep. No.[a] | Antigen dose (Thymocytes) | Route of Injection | Day(s) of Injection | Days of Bleed | No. of Injections | Lympho-cytotoxic Titre $Log_2$[b] |
|---|---|---|---|---|---|---|
| 1a | $12.5 \times 10^9$ | SC in FCA | 0 | 21 | 1 | 6 |
| 1b | $8.9 \times 10^9$ | IV | 28 | 38 | 2 | 11 |
| 1c | $8.6 \times 10^9$ | IV | 99 | 108 | 3 | 14 |
| 1d | $9.7 \times 10^9$ | IV | 148 | 164 | 4 | 11 |
| 2 | $100 \times 10^9$ | IV | 0,14,37,55 and 76 | | | |
| | $9 \times 10^9$ | IV | 136 | | | 10 |
| | $1 \times 10^8$ | SC in FCA | 357 and 820 | 835 | 8 | |
| 3 | 0.5 to $6 \times 10^9$ | SC in FCA | 0,4,7,12 and 14 | | | |
| | $22 \times 10^6$ | SC in FCA | 19,21,28,32, 44 and 49 | | | 8 |
| | $32 \times 10^9$ | SC in FCA | 59 and 63 | | | |
| | $32 \times 10^9$ | IV | 56,66 and 77 | 80 | 16 | |
| 4 | $1 \times 10^8$ | SC in FCA | 0 and 91 | | | |
| | $1 \times 10^8$ | SC in FCA and IV simultan-eously | 210 and 287 | | | |
| | $1 \times 10^8$ | IV | 294,301,308 and 315 | 332 | 8 | 8 |

(a) Antisera 2-4 were kindly provided by Dr. A. Phillips of the Wellcome Research Laboratories, Beckenham, England.

(b) The lymphocytotoxic titre recorded is that noted with a 1g per cent IgG solution prepared from a bleed obtained on the day indicated.

NOTE: Preparations 1d, 2, 3 and 4 significantly prolonged A strain skin grafts on CBA mice.

Preparations 1a, 1b and 1c were not tested.

and dissolved in half a volume of 0.01M phosphate buffer (pH 8.0; see Appendix I). The material was re-precipitated by adding an equal volume of 28 per cent sodium sulphate. The final precipitate was spun down and redissolved in and dialyzed against 0.01M phosphate buffer (pH 7.5; see Appendix I).

Diethyl-amino-ethyl (DEAE) cellulose (Whatman DE.11; exchange capacity 1.0 milli-equivalent per g., obtained from W. & R. Balston, Maidstone, Kent) was washed extensively with 0.01M phosphate buffer (pH 7.5) and resuspended in the same buffer. During the process of washing the fines were removed by decantation. The exchanger was then filtered through a coarse glass sinter, resuspended in the equilibrating buffer and the pH adjusted to 7.5, if necessary, by slow addition of N/1 hydrochloric acid or N/1 sodium hydroxide.

The equilibrated exchanger was added to the gamma globulin solution (obtained by sodium sulphate precipitation) and stirred for ten minutes to ensure adequate mixing. Approximately one gram dry weight (equivalent of 4-5 g. wet weight) of the exchanger was used for each initial ml. of serum. After standing at $4^{\circ}C$ for 30 minutes with occasional stirring, the slurry was filtered through a coarse sintered glass funnel. Entrapped IgG was displaced from the filtered exchanger by the addition of equilibrating buffer and further filtration. The filtrate was concentrated

by lyophilization and redissolving in normal saline.

In order to remove antibodies to mouse erythrocyte and other histocompatibility antigens the anti-lymphocytic globulin was absorbed with mouse erythrocytes. This was achieved by the following procedure. Mouse erythrocytes separated from freshly acquired citrated blood were washed four times with phosphate buffered saline (PBS; see Appendix I). For every five volumes of IgG (2 g. per cent) preparation, one volume of packed mouse erythrocytes were used. Mouse erythrocytes were suspended in the IgG preparation and incubated at 37°C for one hour. The suspension was centrifuged and the IgG solution was recovered. The process was repeated (usually 4-5 times) until the anti-mouse erythrocyte activity was satisfactorily absorbed.

The protein concentration of the IgG preparation was calculated from the ultraviolet absorption at 280mμ using the following formula and adjusted to 2 gram protein per 100 ml.

$$\text{Protein concn. (g. per cent)} = \frac{\text{U.V. absorption (at 280m}\mu\text{) x dilution of the sample}}{\text{Extinction coefficient (i.e. 13.80)}}$$

The standardized IgG preparation was sterilized by ultra-filtration through a 0.22μ millipore membrane and ampouled in small aliquots. The sterility of the products were routinely assessed by the Bacteriology Department, Royal Infirmary, Edinburgh. The final product was stored at

-20°C until required.

Lymphocytotoxicity test

The anti-lymphocytic globulin (ALG) to be tested was adjusted to a concentration of 1.0 g. per cent protein in Hank's balanced salt solution (BSS). Serial dilutions of the preparation were made in Hank's BSS (see Appendix I) to obtain 12-14 tubes of doubling dilutions. To each tube was added 0.2 ml. of dispersed and prewashed (in Hank's BSS) thymocyte suspension containing $10^7$ cells per ml. Finally 0.1 ml. of fresh guinea pig complement diluted 1/10 was added to each tube. The tubes were shaken to mix the cells and incubated at 37°C in a water bath for 1½ hours. Prior to incubation all the reagents and the cell suspension were kept at 4°C. After the incubation, 1.6 ml. of trypan blue solution (see Appendix I) was added to each tube. The tubes were shaken and left at room temperature for 2-3 minutes following which 0.1 ml. of 40 per cent formaldehyde solution was added to all the tubes. The percentage of cells killed by the ALG preparation was estimated either on the same day or the following morning. The cells which had been able to exclude trypan blue were considered live. The dilution at which 50 per cent of cells were killed was taken as the cytotoxicity titre for the ALG preparations.

With each test for cytotoxicity, control tubes lacking

complement or ALG or both were included. The results of the cytotoxicity tests on various ALG preparations used in this study have been summarized in Table 2.

## ANTIGENS

### Sheep erythrocytes (SRBC)

A mixture of sheep blood with an equal volume of Alsever's solution was acquired from Wellcome Reagents Limited, Beckenham, Kent. Prior to administration into mice, or use as an indicator for the assay of the immune responses, the erythrocytes were washed four times with phosphate buffered saline (PBS).

### Purified pneumococcal polysaccharide (type III)

The preparation of the pneumococcal polysaccharide (SSS-III) used as a thymus independent antigen in these studies was kindly provided by Dr. James Howard (Department of Experimental Immunobiology, Wellcome Research Laboratories, Beckenham, Kent) and Dr. J.H. Humphrey (National Institute for Medical Research, Mill Hill, London N.W.7). The detailed method for the preparation of this antigen has been reported elsewhere (Heidelberger, Macleod, Markowitz and Roe, 1950). The product was obtained in a freeze-dried

state and prior to use the antigen was resolublized in normal physiological saline to give the required concentration of the antigen.

Bovine serum albumin (BSA)

A three time crystallized preparation of bovine serum albumin containing 10 mg. protein nitrogen per ml. (i.e. 63 mg. total protein per ml.) was obtained from the Armour Pharmaceutical Company, Chicago, Illinois, U.S.A. Prior to administration into mice the antigen was adsorbed onto 'Alhydrogel' (1.3 per cent $Al_2O_3$) supplied by Danok Svovlsyr, Copenhagen, Denmark. To achieve the adsorption, 1.6 ml. of the standard BSA solution (100 mg. protein) was added to 10 ml. of well suspended Alhydrogel and mixed adequately. The mixture was incubated at 37°C for half an hour. Following this the mixture was spun at top speed in an MSE-Minor bench centrifuge for 20 minutes and the protein-free supernatant was discarded. The pellet was resuspended in an appropriate volume of sterile physiological saline to give the required concentration of the protein per ml.

INJECTION OF ANIMALS

Normal horse IgG (NHIgG) or ALG were injected intra-peritoneally as a sterile solution containing 5.0 mg. IgG protein in 0.2-0.3 ml. volume. Unless otherwise mentioned

ALG 1d was used in all the experiments. In selected experiments other ALG preparations were used.

The appropriate dose of SRBC was administered intraperitoneally in 0.4 ml. PBS.

The required dose of purified SSS-III was injected intravenously in 0.2 ml. saline via the tail vein. To facilitate intravenous administration the mice to be injected were incubated at 40°C for about 10 minutes prior to the injection.

Alum precipitated BSA was injected intraperitoneally as Alhydrogel adsorbed suspension in 0.2 ml. sterile physiological saline.

## ANTI-IMMUNOGLOBULIN SERA

### Anti-mouse whole gamma globulin serum

Mice were immunized with $10^9$ rabbit erythrocytes previously washed with PBS. Two weeks later the mice were bled and the serum was inactivated by heating at 56°C for 30 minutes. 0.5 ml. washed and packed rabbit erythrocytes were suspended in 2 ml. of this mouse anti-rabbit serum and incubated at 37°C for half an hour. The rabbit erythrocytes thus coated with mouse gamma globulin were washed six times with PBS, emulsified in Freund's complete adjuvant and injected intramuscularly into the hind leg of

the rabbit from which the erythrocytes were initially obtained. Three weeks later the rabbit was boosted with similarly sensitized autologous erythrocytes by intravenous injection. The antiserum obtained a week later was inactivated by heating at 56°C for 30 minutes, and stored at -20°C. Prior to use as a developing serum for indirect anti-sheep erythrocyte plaques the antiserum was absorbed with sheep erythrocytes and titrated for optimum dilution for developing the indirect plaques. The procedures for determining the optimum dilution of the antiserum in the plaque forming cell assay will be described later.

Antisera against individual mouse immunoglobulins

The antisera against different IgG subclasses ($IgG_1$, $IgG_{2a}$, $IgG_{2b}$) and IgA were kindly provided by Professor Stewart Sell (Department of Pathology, University of California Medical School, San Diego, California, U.S.A.). These antisera were raised in rabbits, and the details of the antigens used to immunize the rabbits have been listed in Table 3. The details of preparation of antigens and the schedule of immunization have been documented elsewhere (Sell, Park and Nordin, 1970). These antisera were used for detecting the cells producing anti-sheep erythrocyte haemolysins of the respective immunoglobulin classes and subclasses. Before use each antiserum was titrated for its optimal developing titre.

TABLE 3 - IMMUNOGENS USED FOR THE PREPARATION OF ANTI-MOUSE IMMUNOGLOBULIN SUBCLASS SERA[a]

| Immunoglobulin Class | Immunogenic Fragment | Source of Antigen (Myeloma) |
|---|---|---|
| $\gamma_1$ | Fc | MOPC-70A |
| $\gamma_{2a}$ | Fc | RPC-5 |
| $\gamma_{2b}$ | Fc | MPC-11 |
| YA | Fc | MPC-40 |

(a) See Sell, Park and Nordin (1970) for details.

## ASSAY PROCEDURE FOR THE IMMUNE RESPONSES

The experimental animals were anaesthetized in ether. The maximum possible amount of blood was drawn from the inferior vena cava. The intact spleens were carefully excised from mice which had been immunized with sheep erythrocyte or type III pneumococcal polysaccharide. These spleens were immediately used for the plaque forming cell assay, whereas the sera separated from the blood were stored at -20°C until they were assayed for circulating antibody titres against the respective antigens. In certain experiments circulating antibody titres in individual mice were determined at periodic intervals following antigenic challenge. On these occasions 0.3 to 0.4 ml. of blood was drawn from the retro-orbital sinus at intervals. The serum was separated on the same day and stored at -20°C prior to assay.

### Anti-sheep erythrocyte response

The antibody plaque forming cell (PFC) assay was mainly used to assess the anti-sheep erythrocyte responses. Apart from this, the sera from all the animals challenged with this antigen were also tested for anti-sheep erythrocyte haemagglutinating and haemolytic antibody titres. Haemolysin and haemagglutinin assays were performed on native as well as 2-mercaptoethanol-treated serum. Both PFC and serum antibody assays were carried out on individual mice.

Plaque forming cell assay

The spleen from the mice to be tested was removed and gently disrupted by means of a glass homogenizer in Eagle's medium (Wellcome Reagents Limited) containing 100 mg. BSA per 100 ml. The dispersed cells were counted to determine the total number of nucleated cells per spleen. The cells were well suspended and left to settle for 5 minutes. An aliquot of the suspension was taken from the top of the tube and made up to 6 ml. with the BSA-supplemented Eagle's medium. The cells were suspended by gently shaking, and spun at 750 r.p.m. (150g) in an MSE Mistral-6 centrifuge for 6-7 minutes. The supernatant was discarded and the cells were resuspended in an appropriate volume of the medium (usually 2 ml.). The suspension was allowed to stand for another 5 minutes and the upper layer of the suspension containing singly dispersed cells was carefully removed. The cell count of this suspension was determined and if necessary it was adjusted to the required concentration. The concentration of cells in the suspension varied from experiment to experiment and also from group to group in the same experiment depending upon the anticipated plaque forming cell response of the animals under test. This was mainly governed by the strain of animals, the antigen dose and the treatment the animals had received (e.g. NHIgG-treated animals were anticipated to give far bigger response than ALG-treated ones). The PFC assay

on test (treated with ALG) and the control (treated with NHIgG) animals were invariably performed at the same time. Throughout these operations the cells were kept at 4°C.

The number of antibody PFC were determined by a modification of the Jerne plaque technique (Jerne, Nordin and Henry, 1963). A stock solution of 1.8 per cent (w/v) Agarose A-37 Indubois$^{R}$ (L'Industrie Biologique Francais, Gennevilliers, France) in distilled water was melted by heating at 100°C in a boiling water bath. This was then cooled down to 48°C and mixed with an equal volume of double strength prewarmed (48°C) Eagle's medium supplemented with 200 mg. BSA per 100 ml. The mixture which was maintained at 48°C in a water bath was then filtered through glass wool which was soaked immediately before use with warm (48°C) Eagle's medium. The filtered agarose was collected in a prewarmed container. The mixture was dispensed in one ml. aliquots in prewarmed Kahn tubes using a prewarmed 5 ml. pipette. To this was added 0.1 ml. of 10per cent (v/v) prewashed sheep erythrocyte suspension in Hank's balanced salt solution and 0.1 ml. of spleen cell suspension from the animals to be tested. The number of spleen cells per plate were chosen such that not more than 200 plaques were produced per plate. The cells were mixed by gently stirring the suspension with a wooden applicator kept in Eagle's medium at 48°C. They were then poured into a 5 cm. diameter disposable plastic

Petri dish (Sterilin Limited, Richmond, Surrey) laid on a level surface. The mixture was quickly spread over the whole area of the plate and left to set. Tests on each spleen were performed in duplicate. The plates were placed in a chromatography tank filled with 5 per cent $CO_2$ in air and incubated at 37°C for two hours. Following this, 2 ml. of a 1/40 dilution of preserved guinea pig complement (Wellcome Reagents Limited) in Hank's BSS was poured gently over the gel and the plates incubated for another hour at 37°C. The complement was then poured off and the plates were either stored at 4°C in which case the haemolytic plaques were read the following morning or they were covered with 1 ml. of 0.25 per cent (v/v) glutaraldehyde, stored at 4°C and read within 2-3 days. To facilitate the counting of the plaques, the plates were placed on a grid and a hand lens was used to magnify the plaques.

Since the direct plaques only represented the IgM producing cells, the cells producing other antibody classes, were detected by the use of a developing technique described by Dresser and Wortis (1967). This involved the addition of a suitable dilution of a multivalent or a monovalent rabbit anti-mouse immunoglobulin serum with the complement. As most antiglobulin sera inhibit IgM producing plaques to some degree, it was necessary to determine the corrected numbers of the developed plaques using the developing and

the inhibiting constants for the antiglobulin sera at the dilution used. These were determined as follows.

Titration of the developing antisera

A/HeJ mice were injected intraperitoneally with a low dose ($3 \times 10^7$) of sheep erythrocytes. Two days following this they were sacrificed and the spleens were assayed for direct plaque forming cells as well as the indirect plaque forming cells, the latter being developed with various dilutions of the anti-immunoglobulin serum to be titrated. Since the antibody response against this dose of sheep erythrocytes at this time after immunization is mainly IgM, any decrease in the plaque forming cells obtained within a constant number of spleen cells by the incorporation of the anti-immunoglobulin serum would represent the net inhibition of the IgM plaques by the antiserum dilution. Thus the inhibition constants (KI) for various dilutions of the antiserum were calculated by the following formula:

$$KI_{(\text{at the dilution})} = \frac{\text{developed plaques (at this dilution)}}{\text{undeveloped (direct) plaques}}$$

Calculation of the developing constant and the optimum dilution of the antiserum: Mice were immunized with $3 \times 10^9$ sheep erythrocytes and sacrificed for the PFC assay ten days later. Alternatively mice were primed with $3 \times 10^7$ and

a fortnight later they were challenged with $3 \times 10^8$ sheep erythrocytes. The spleen cells from the latter group were tested for PFC 4 days after the secondary challenge. In both these cases in addition to a small IgM response the mice gave considerable IgG and IgA response (Wortis, Dresser and Anderson, 1969; Sell, Park and Nordin, 1970). The spleens of the above mice were assayed for direct PFC using no developing serum and indirect PFC using different dilutions of the anti-immunoglobulin serum to be tested. From this the optimum dilution of the antiserum to be used for developing the indirect PFC was determined. In addition the developing constants (KD) for the respective anti-immunoglobulin at its optimum dilution of use were calculated by the following formula:

$$KD_{(\text{opt. dil.})} = \frac{\text{Max. dev. plaques} - \text{undev. plaques} \times \text{KI (at this dilution)}}{\text{Dev. plaques (at opt. dil.)} - \text{undev. plaques} \times \text{KI (at opt. dil.)}}$$

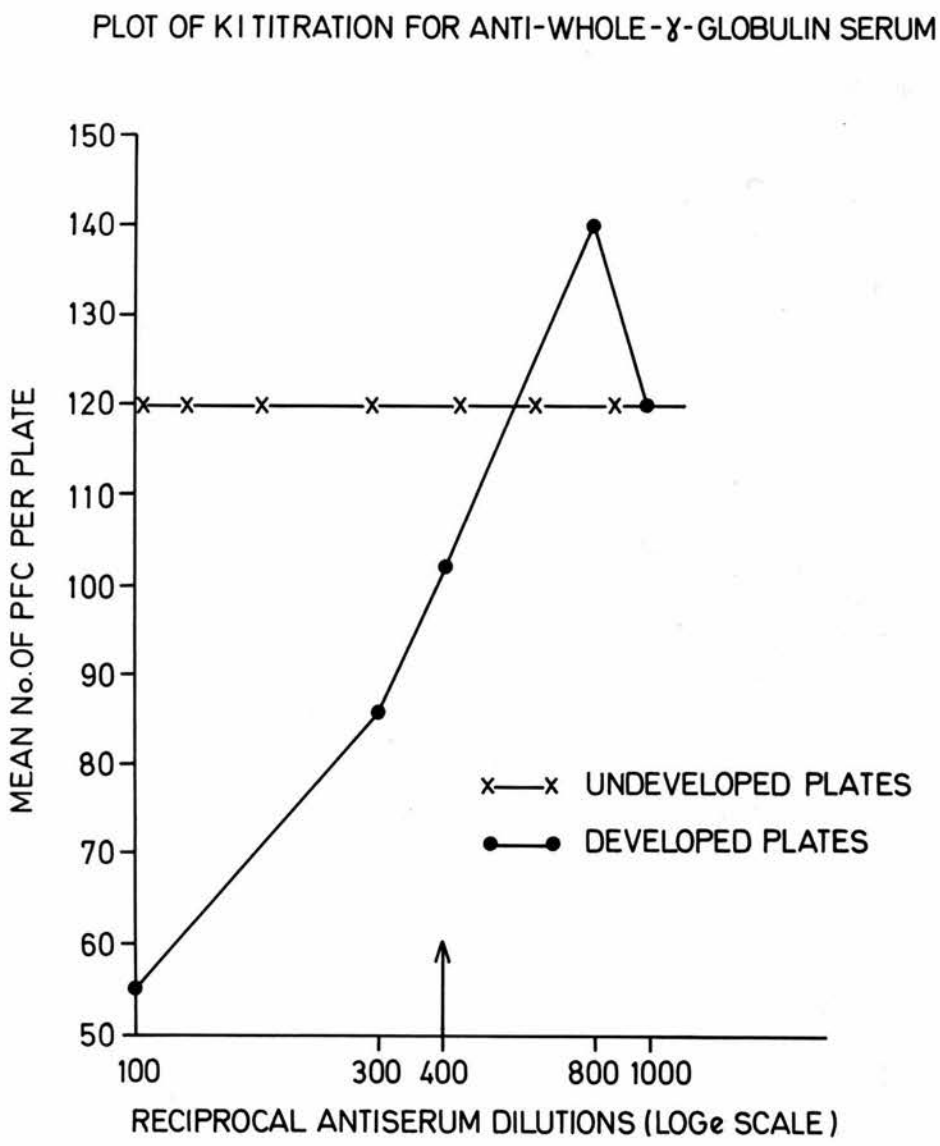
Using the inhibition and the developing constant values the corrected indirect plaques were determined by the following formula:

$$\text{Corrected PFC} = KD(\text{dev. plaques} - \text{undev. plaques} \times KI)$$

(both KD and KI values were those for the optimum dilution of the developing serum used)

The results of the titration of various anti-immunoglobulin sera have been shown in Figures 1-5; the optimum dilutions

FIGURE 1


PLOT OF KI TITRATION FOR ANTI-WHOLE-γ-GLOBULIN SERUM
MEAN No.OF PFC PER PLATE
150
140
130
120
110
100
90
80
70
60
50
100
300 400
800 1000
RECIPROCAL ANTISERUM DILUTIONS (LOGe SCALE)
x—x UNDEVELOPED PLATES
DEVELOPED PLATES

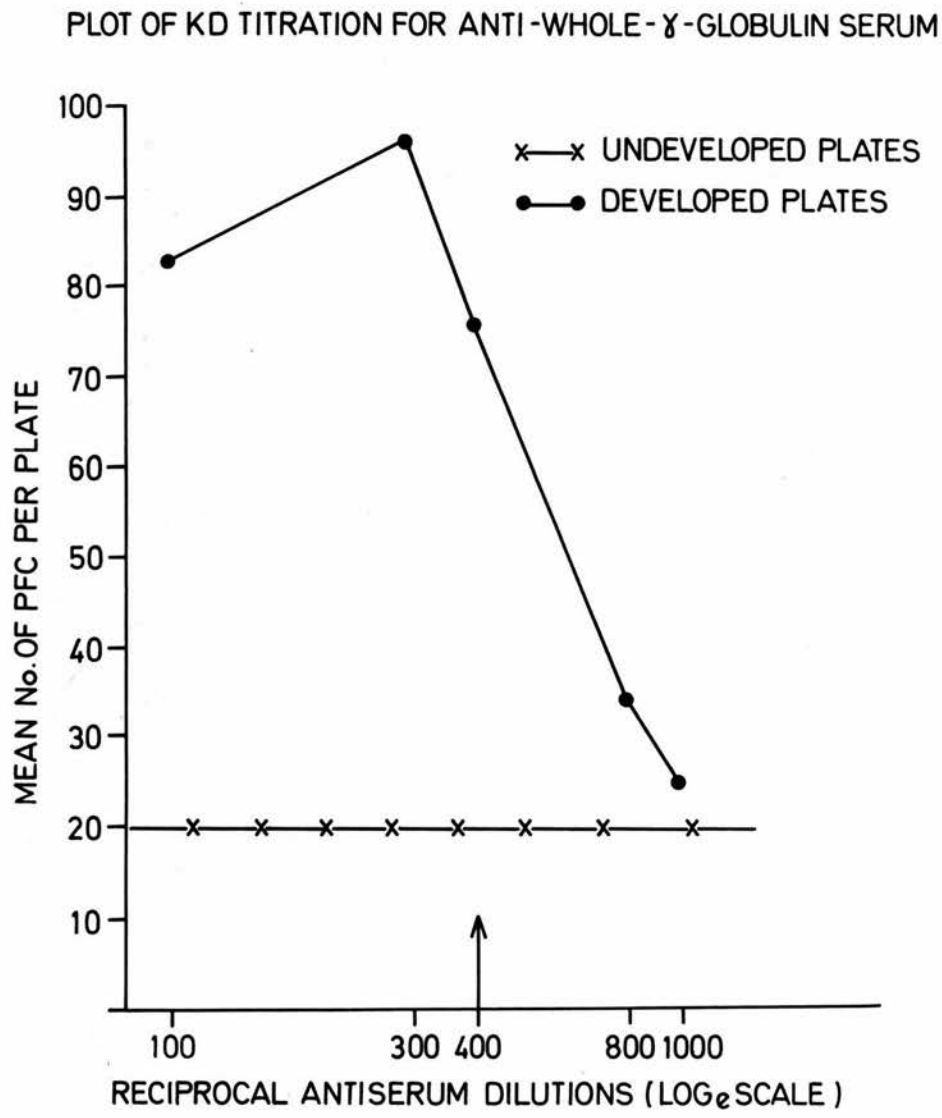
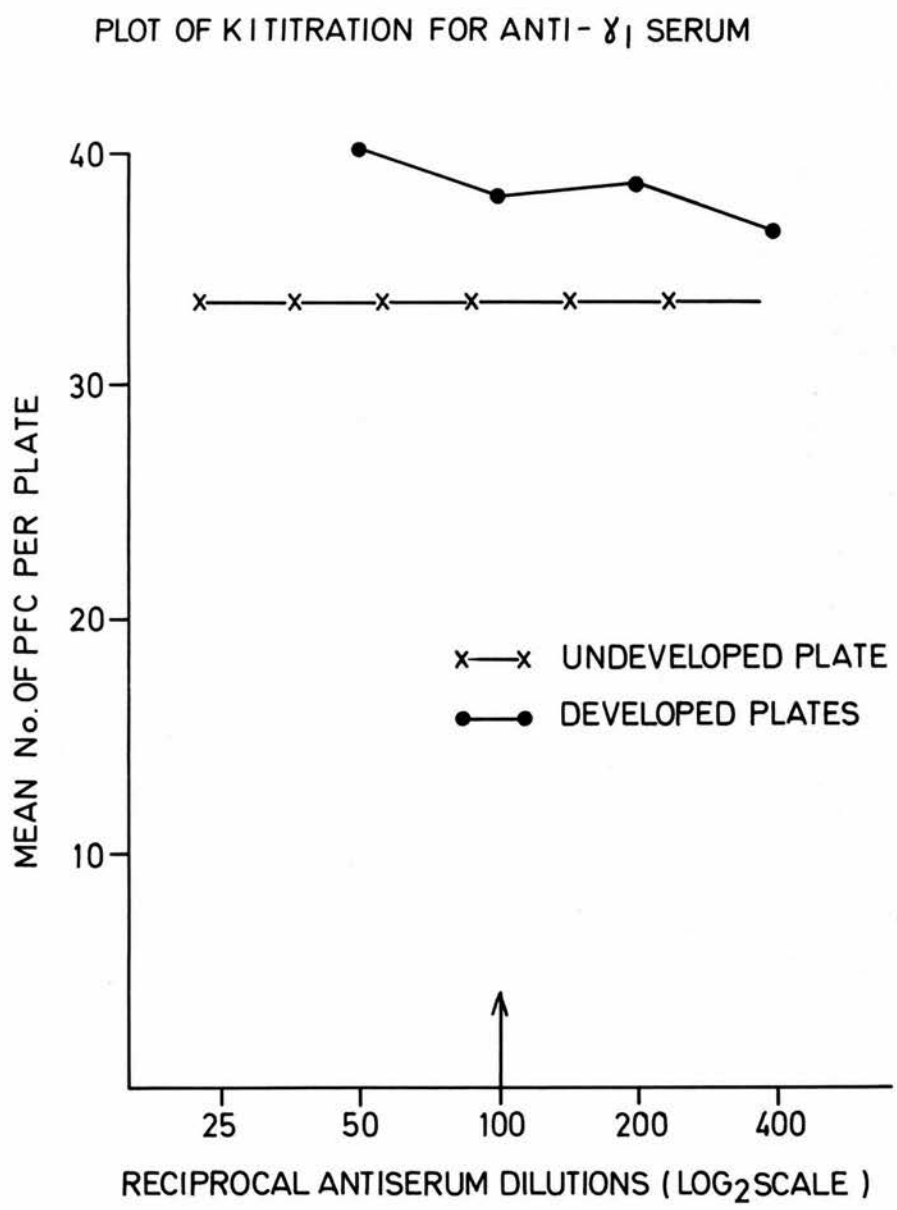

PLOT OF KD TITRATION FOR ANTI-WHOLE-γ-GLOBULIN SERUM
MEAN No.OF PFC PER PLATE
100
90
80
70
60
50
40
30
20
10
100
300 400
800 1000
RECIPROCAL ANTISERUM DILUTIONS (LOGe SCALE)
x—x UNDEVELOPED PLATES
DEVELOPED PLATES

FIGURE 2


PLOT OF K I TITRATION FOR ANTI - γ1 SERUM
MEAN No. OF PFC PER PLATE
40
30
20
10
x—x UNDEVELOPED PLATE
DEVELOPED PLATES
25
50
100
200
400
RECIPROCAL ANTISERUM DILUTIONS ( LOG2 SCALE )

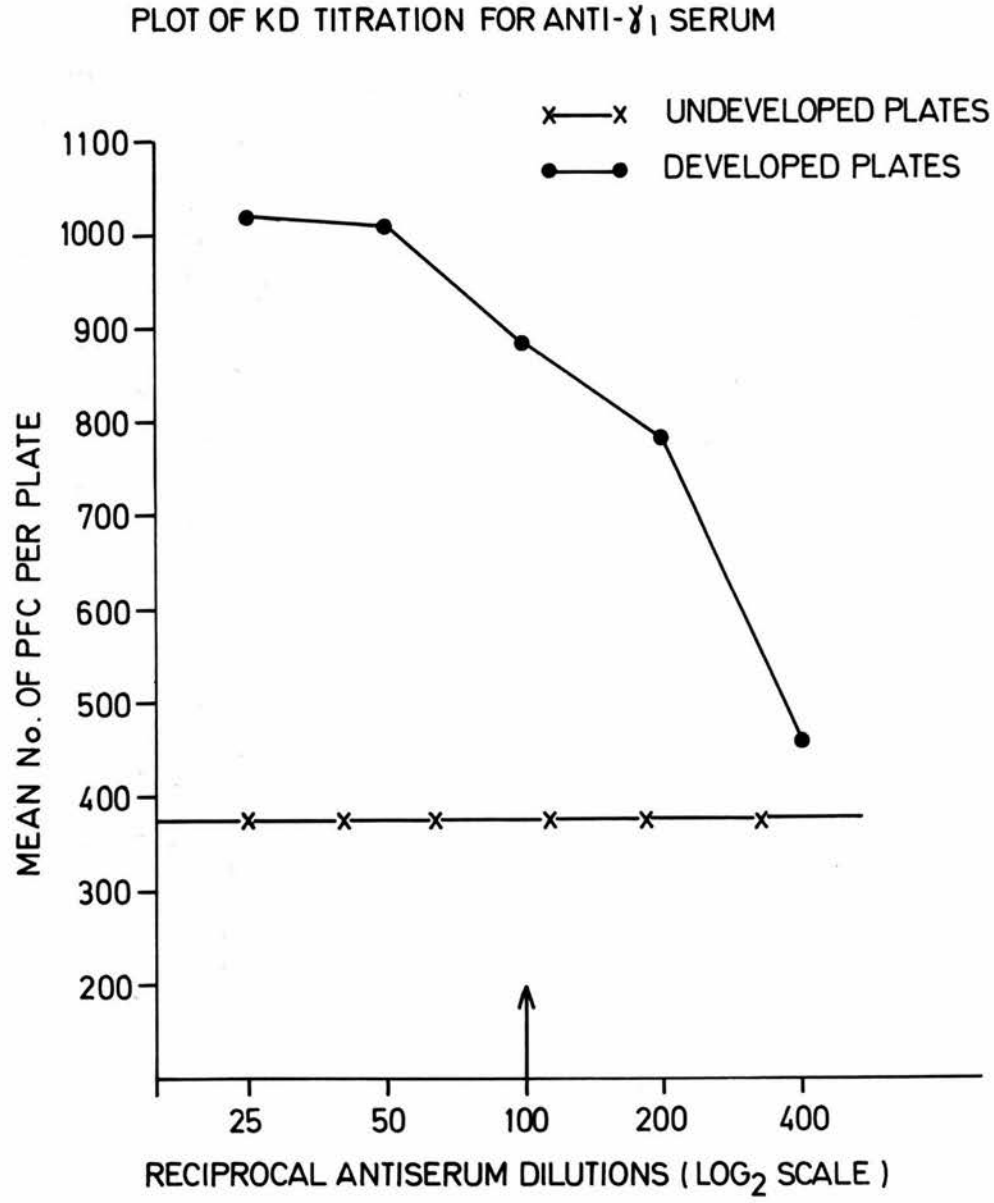

PLOT OF KD TITRATION FOR ANTI-γ1 SERUM
x—x UNDEVELOPED PLATES
DEVELOPED PLATES
MEAN No. OF PFC PER PLATE
1100
1000
900
800
700
600
500
400
300
200
25
50
100
200
400
RECIPROCAL ANTISERUM DILUTIONS ( LOG2 SCALE )

FIGURE 3

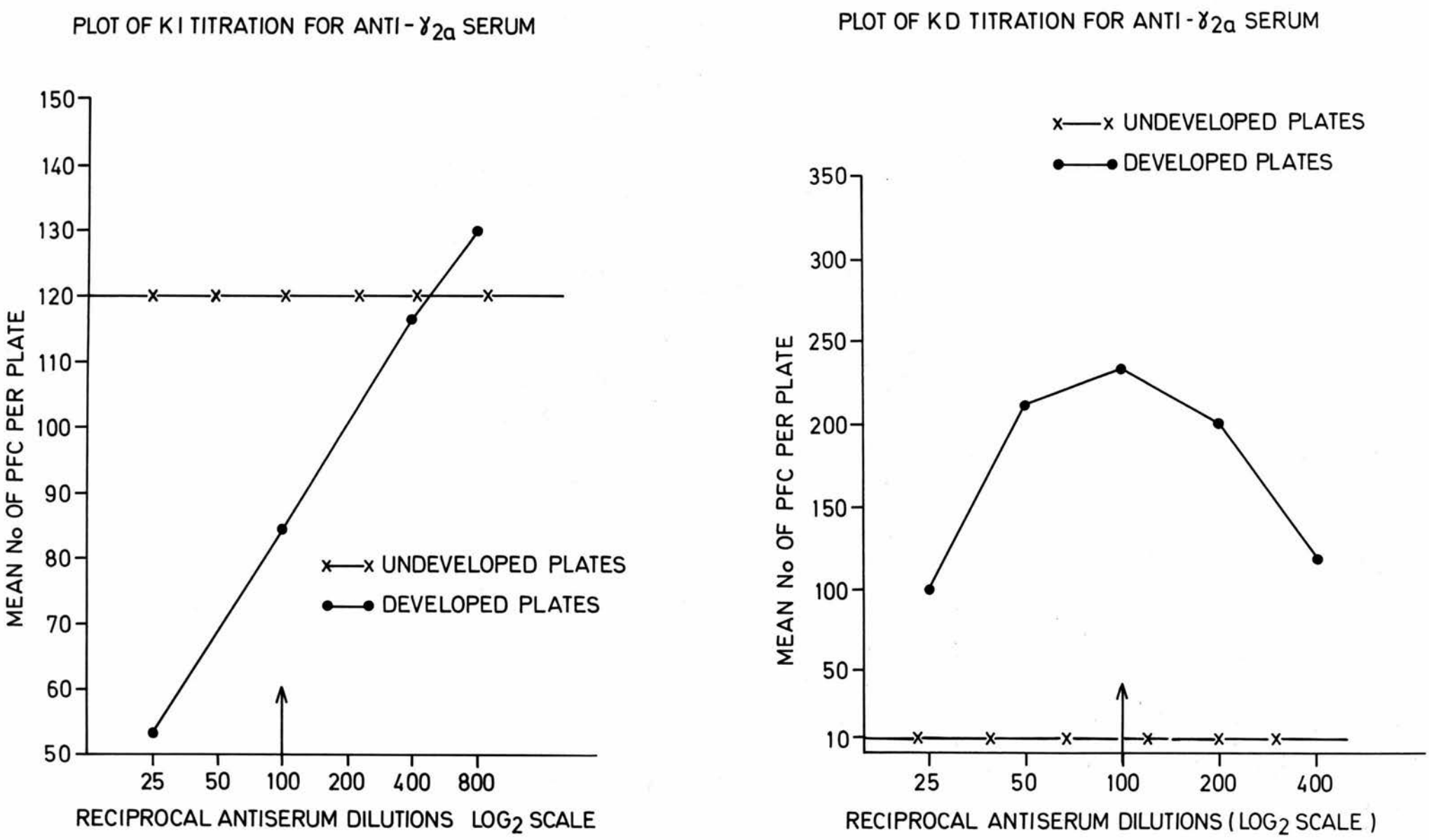
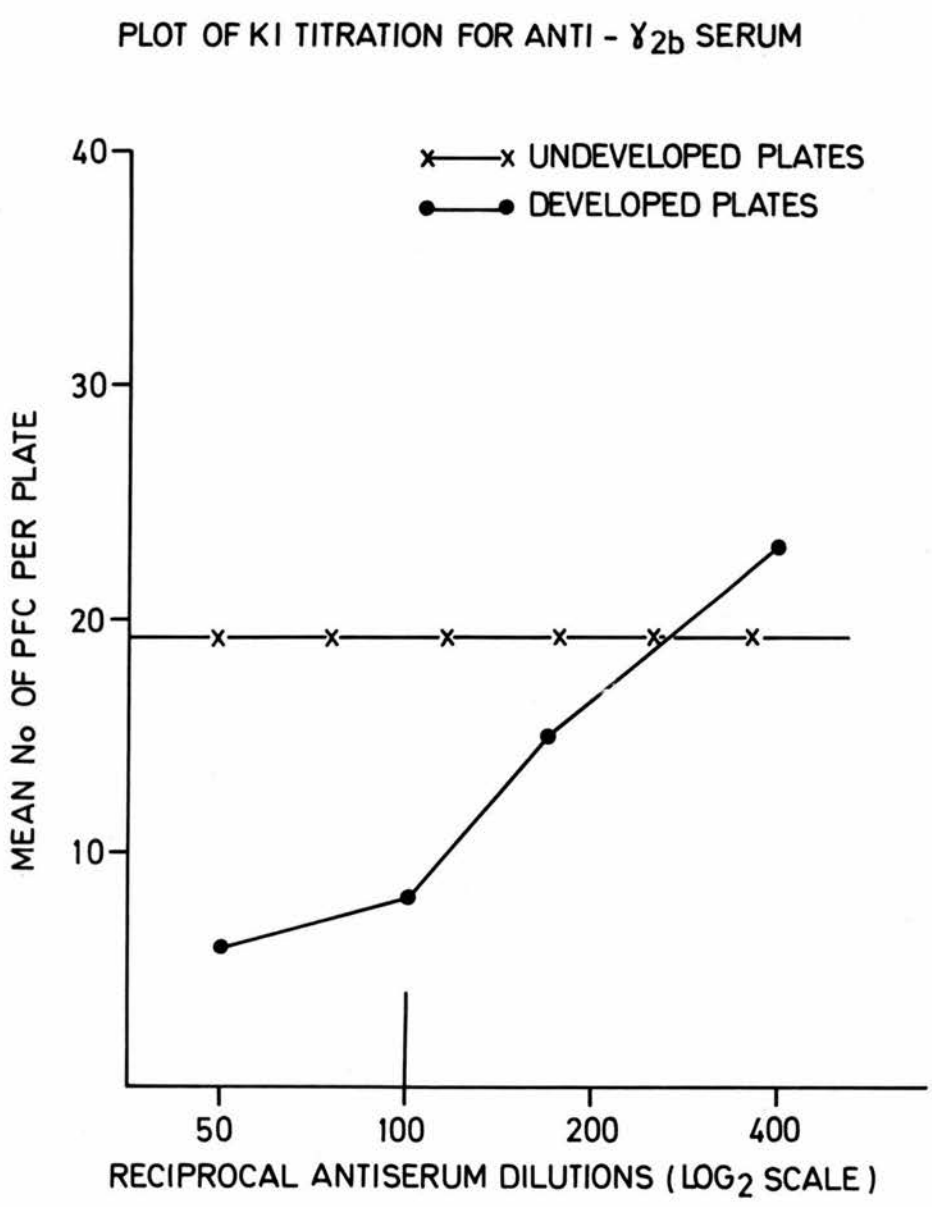

PLOT OF K I TITRATION FOR ANTI - γ2a SERUM
MEAN No OF PFC PER PLATE
150
140
130
120
110
100
90
80
70
60
50
x—x UNDEVELOPED PLATES
DEVELOPED PLATES
25
50
100
200
400
800
RECIPROCAL ANTISERUM DILUTIONS LOG2 SCALE
PLOT OF K D TITRATION FOR ANTI - γ2a SERUM
x—x UNDEVELOPED PLATES
DEVELOPED PLATES
MEAN No OF PFC PER PLATE
350
300
250
200
150
100
50
10
25
50
100
200
400
RECIPROCAL ANTISERUM DILUTIONS ( LOG2 SCALE )

FIGURE 4


PLOT OF KI TITRATION FOR ANTI - γ2b SERUM
x—x UNDEVELOPED PLATES
●—● DEVELOPED PLATES
MEAN No OF PFC PER PLATE
40
30
20
10
50
100
200
400
RECIPROCAL ANTISERUM DILUTIONS (LOG2 SCALE)

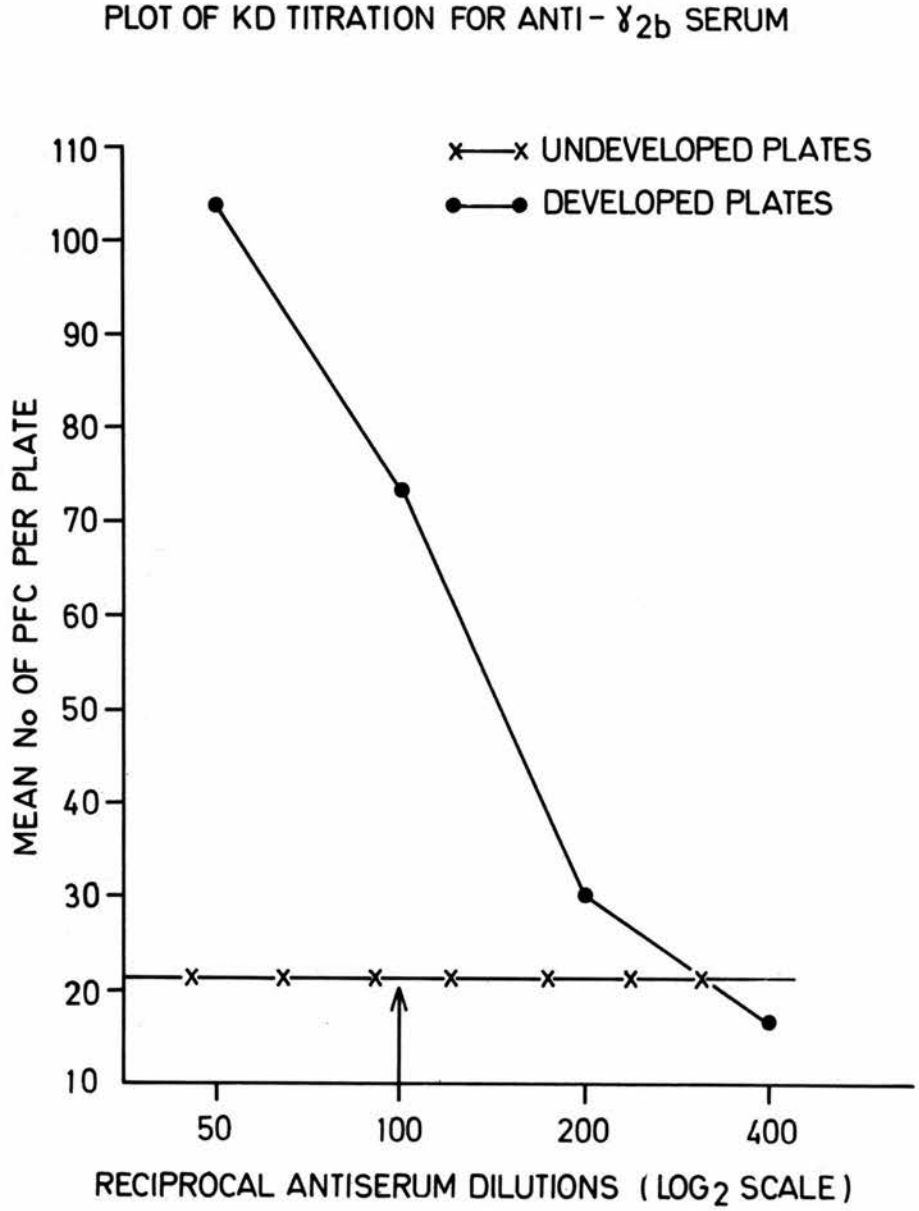
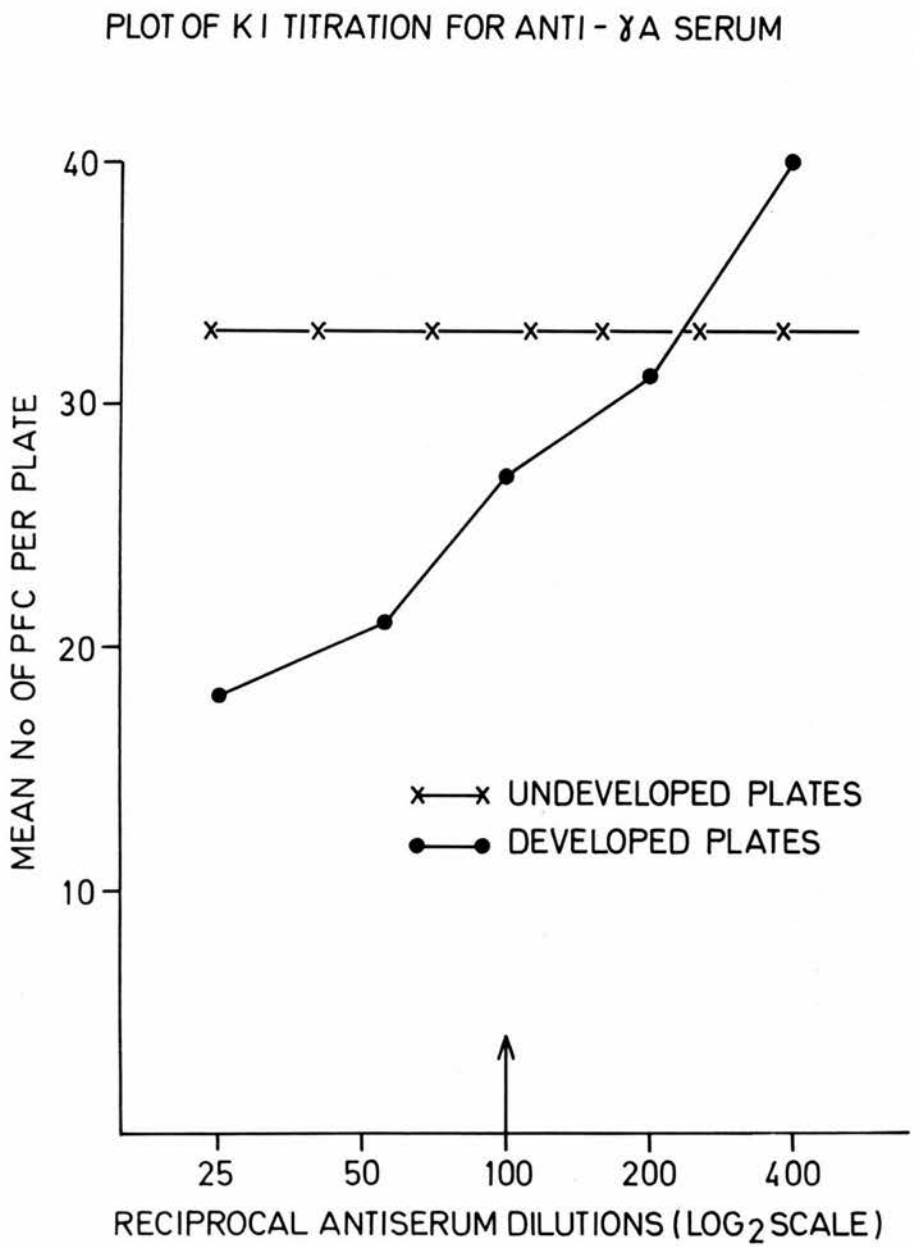
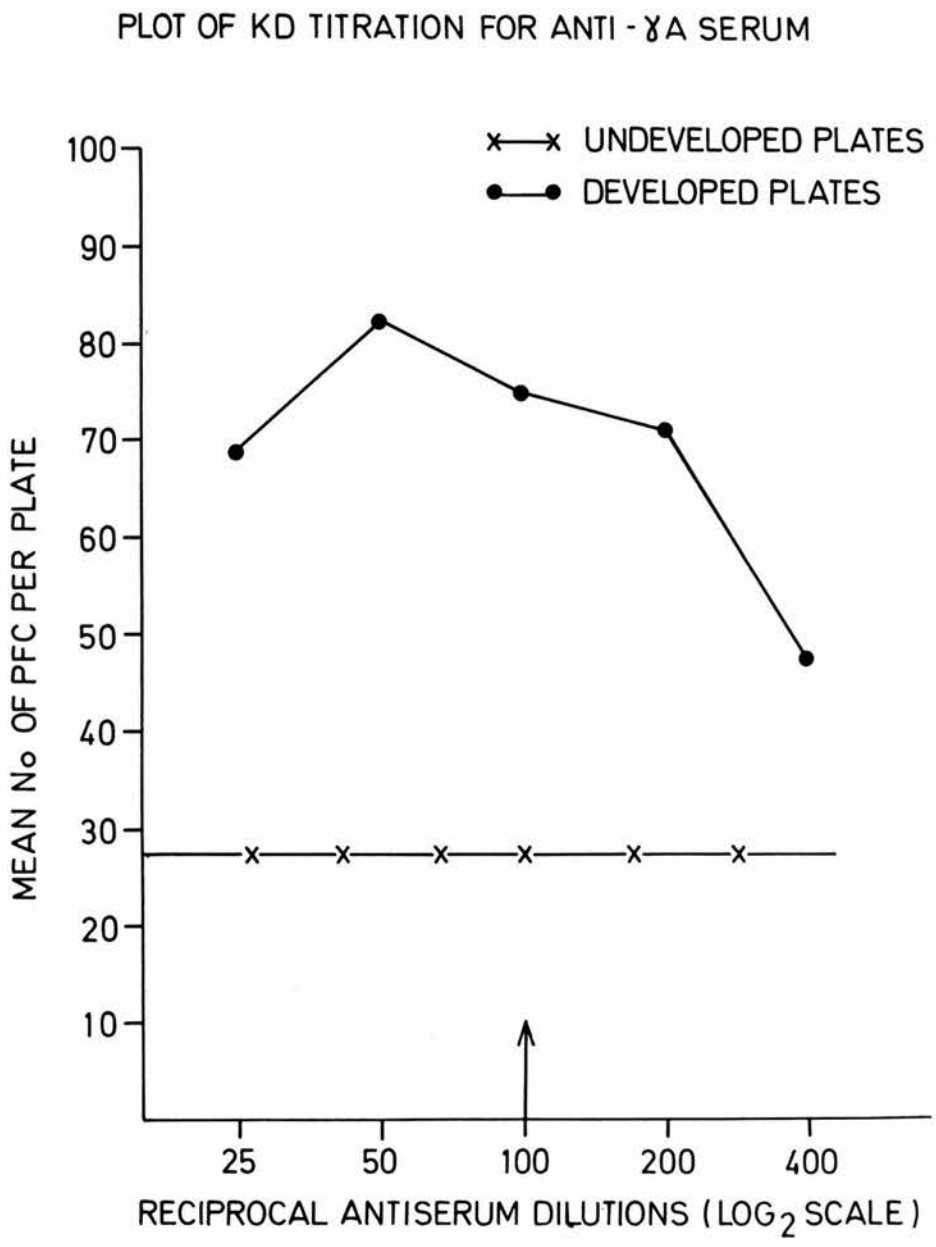

PLOT OF KD TITRATION FOR ANTI - γ2b SERUM
x—x UNDEVELOPED PLATES
●—● DEVELOPED PLATES
MEAN No OF PFC PER PLATE
110
100
90
80
70
60
50
40
30
20
10
50
100
200
400
RECIPROCAL ANTISERUM DILUTIONS (LOG2 SCALE)

FIGURE 5


PLOT OF K I TITRATION FOR ANTI - γA SERUM
MEAN No OF PFC PER PLATE
40
30
20
10
UNDEVELOPED PLATES
DEVELOPED PLATES
25
50
100
200
400
RECIPROCAL ANTISERUM DILUTIONS (LOG2 SCALE)


PLOT OF KD TITRATION FOR ANTI - γA SERUM
MEAN No OF PFC PER PLATE
100
90
80
70
60
50
40
30
20
10
UNDEVELOPED PLATES
DEVELOPED PLATES
25
50
100
200
400
RECIPROCAL ANTISERUM DILUTIONS (LOG2 SCALE)

used, and the inhibition and developing constants for these antisera have been recorded in Table 4.

Haemagglutination test: Doubling dilutions of the sera under test were made in PBS in V-shaped microtitre plates (Sterilin) using microtitre dispensing pipettes and loops (Cooke Engineering Company, Alexandria, Virginia, U.S.A.). An equal volume (0.025 ml.) of 2 per cent (v/v) sheep erythrocyte suspension in PBS was added and mixed. The plates were covered with sellotape, in order to avoid evaporation, and incubated at 37°C for one hour. At the end of the incubation the plates were carefully shaken to resuspend the cells and stored at 4°C overnight. The agglutinin titres were read macroscopically on the following morning. A button of packed cells half the size of the control (the well containing saline and sheep erythrocytes) was taken as the end-point.

Haemolysin assay: The sera were serially diluted (2-fold) in veronal buffered saline (VBS; see Appendix I) in U-shaped microtitre plates (Sterilin) using the 0.025 ml. microtitre dispensing pipette and loops. An equal volume of 2 per cent (v/v) sheep erythrocyte suspension in VBS was added and mixed. The plates were incubated at 37°C for 30 minutes, following which the cells were resuspended and 0.025 ml. preserved guinea pig complement (Wellcome) at a 1/15 dilution was added. The plates were reincubated

TABLE 4 - INHIBITION (KI) AND DEVELOPING (KD) CONSTANTS FOR ANTI-IMMUNOGLOBULIN SERA AND THE DILUTIONS USED

| Antiserum | Dilution used | KI | KD |
|---|---|---|---|
| anti-whole immunoglobulin | 1/400 | 0.85 | 1.40 |
| anti-$\gamma_1$ | 1/100 | 1.0 | 1.55 |
| anti-$\gamma_{2a}$ | 1/100 | 0.72 | 1.0 |
| anti-$\gamma_{2b}$ | 1/100 | 0.40 | 1.51 |
| anti-YA | 1/100 | 0.79 | 1.24 |

at 37°C for another hour. The plates were shaken at the beginning, in the middle and at the end of the incubation to resuspend the cells. The plates were stored at 4°C overnight and the 50 per cent haemolytic titres were read on the following morning.

2-Mercaptoethanol treatment: Whenever the determination of 7S anti-sheep erythrocyte haemagglutinin or haemolysin titres were desired, the sera were mixed with an equal volume (0.025 ml.) of 0.2M 2-mercaptoethanol in PBS in microtitre plates and left overnight at 4°C. The treated sera were then diluted 2-fold and tested for agglutinin and haemolysin titres as described above.

Anti-pneumococcal polysaccharide response

Anti-SSS-III response was measured by assaying the splenic plaque forming cells. In addition, sera from these mice were tested for haemagglutinating antibodies. In the PFC assay, sheep erythrocytes coated with pneumococcal polysaccharide (a crude preparation) were used as indicator cells, while in the haemagglutination assays the polysaccharide antigen was attached to mouse erythrocytes.

Preparation of crude pneumococcus polysaccharide filtrate: The crude type III pneumococcus polysaccharide filtrate used to sensitize sheep or mouse erythrocytes was prepared by a method based on that described by Askonas,

Farthing and Humphrey (1960). Freeze-dried culture of Diplococcus pneumoniae type III was obtained from the National Collection of Typed Culture (NCTC), Central Public Laboratory, Colindale, London. The contents of the vial were suspended in 5 ml. of brain-heart infusion broth (Difco Laboratories, Detroit 1, Michigan, U.S.A.) and incubated at 37°C overnight. The organisms were subcultured on a blood agar plate and incubated at 37°C for another 16-18 hours. At this stage the purity of the culture was checked by using Optochin (ethyl hydrocuprein-HCL) sensitivity discs (Mast Laboratories). Individual pneumococcus colonies were picked up from the blood agar plate and inoculated into 10 ml. of brain-heart infusion broth. After 24 hours of incubation the brain-heart infusion broth was added to 100 ml. of the protein-free broth (see Appendix I) described by O'Meara and Brown (1936) and incubated at 37°C for 36 to 40 hours.

Formaldehyde (final concentration 1.6 per cent v/v) was then added to the cultures grown in the protein-free broth and the mixture was left at 4°C for 48 hours. The organisms were removed by centrifugation at 6000 r.p.m. (3000g) in an MSE 'high speed 18' centrifuge. The supernatant fluid was recovered and dialysed against a total of 40 volumes of PBS at 4°C with at least four changes of the buffer. The filtrate was titrated for its optimum sensitizing ability in both PFC and haemagglutination

assays and stored in 5 ml. aliquots at -20°C until required. The results of the titrations are shown in Figures 6-7.

Sensitization of sheep erythrocytes: Sheep erythrocytes (obtained from Wellcome Reagents) were washed four times with PBS and made up to a 10 per cent (v/v) suspension. An optimal volume of the crude filtrate was made up to 9 ml. in PBS and to this 1 ml. of 10 per cent sheep erythrocyte suspension was added and quickly mixed. The mixture was incubated at 37°C for one hour with frequent mixing. The sensitized cells were washed four times with PBS and resuspended in Hank's BSS at a 10 per cent (v/v) concentration.

Plaque forming cell assay: The plaque forming cells were assayed using sheep erythrocytes sensitized with polysaccharide as described above. The method adopted for the detection of the anti-SSS-III PFC was essentially the same as that described for the detection of anti-sheep erythrocyte PFC except that in this assay preserved guinea pig complement was replaced by fresh guinea pig serum. The spleens from individual animals were assayed in duplicate for plaque forming cells against both sensitized and unsensitized sheep erythrocytes. The specific anti-SSS-III PFC response in each spleen was calculated by deducting the plaques obtained with unsensitized sheep erythrocytes from those obtained with sensitized erythrocytes. The specificity of the assay was ascertained by the inhibition of the

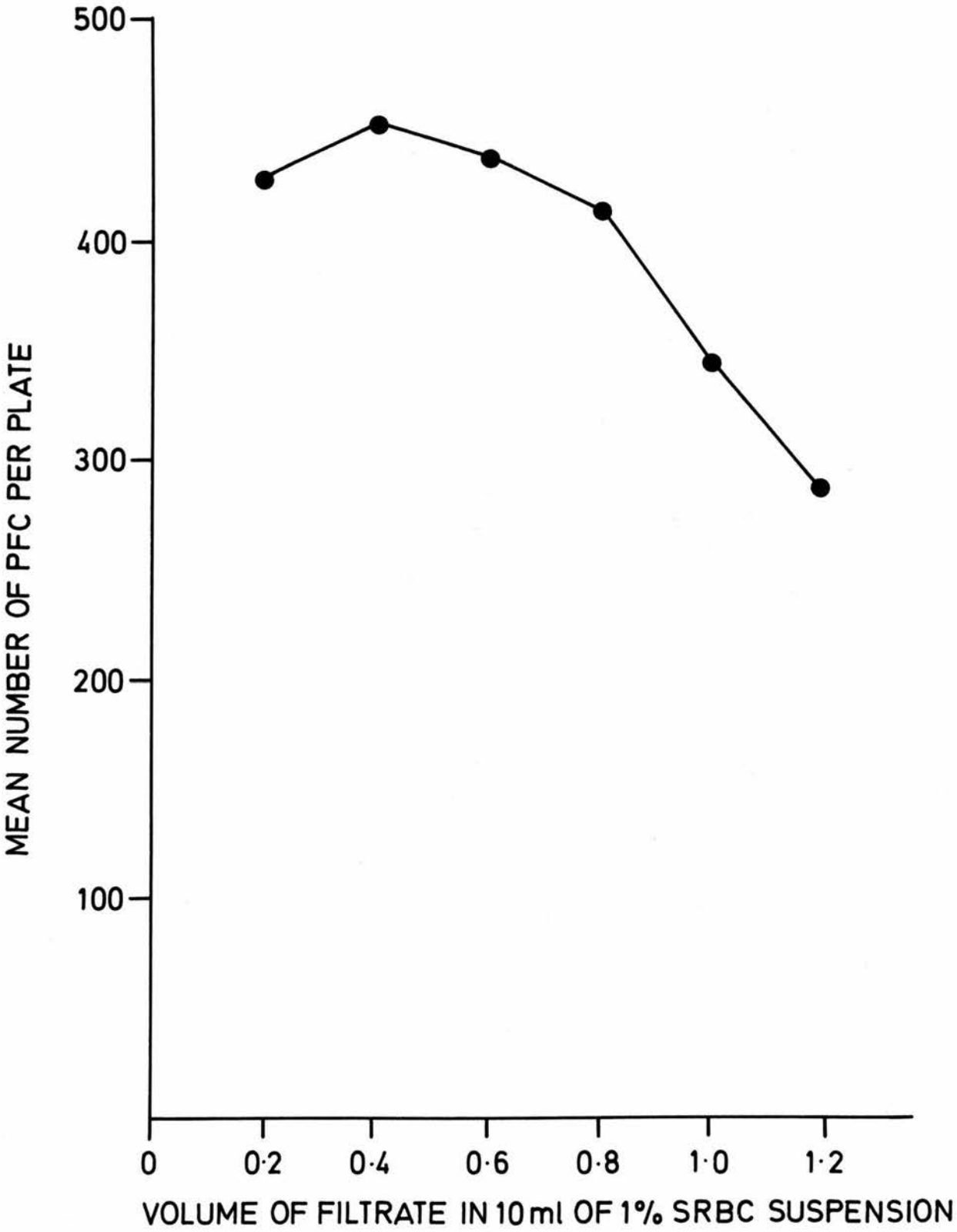

TITRATION OF PNEUMOCOCCAL FILTRATE TO SENSITIZE SHEEP ERYTHROCYTES (SRBC) FOR PFC ASSAY
MEAN NUMBER OF PFC PER PLATE
500
400
300
200
100
0
0·2
0·4
0·6
0·8
1·0
1·2
VOLUME OF FILTRATE IN 10 ml OF 1% SRBC SUSPENSION

FIGURE 6

TITRATION OF PNEUMOCOCCAL FILTRATE TO SENSITIZE MOUSE ERYTHROCYTES (MRBC) FOR HAEMAGGLUTINATION ASSAY

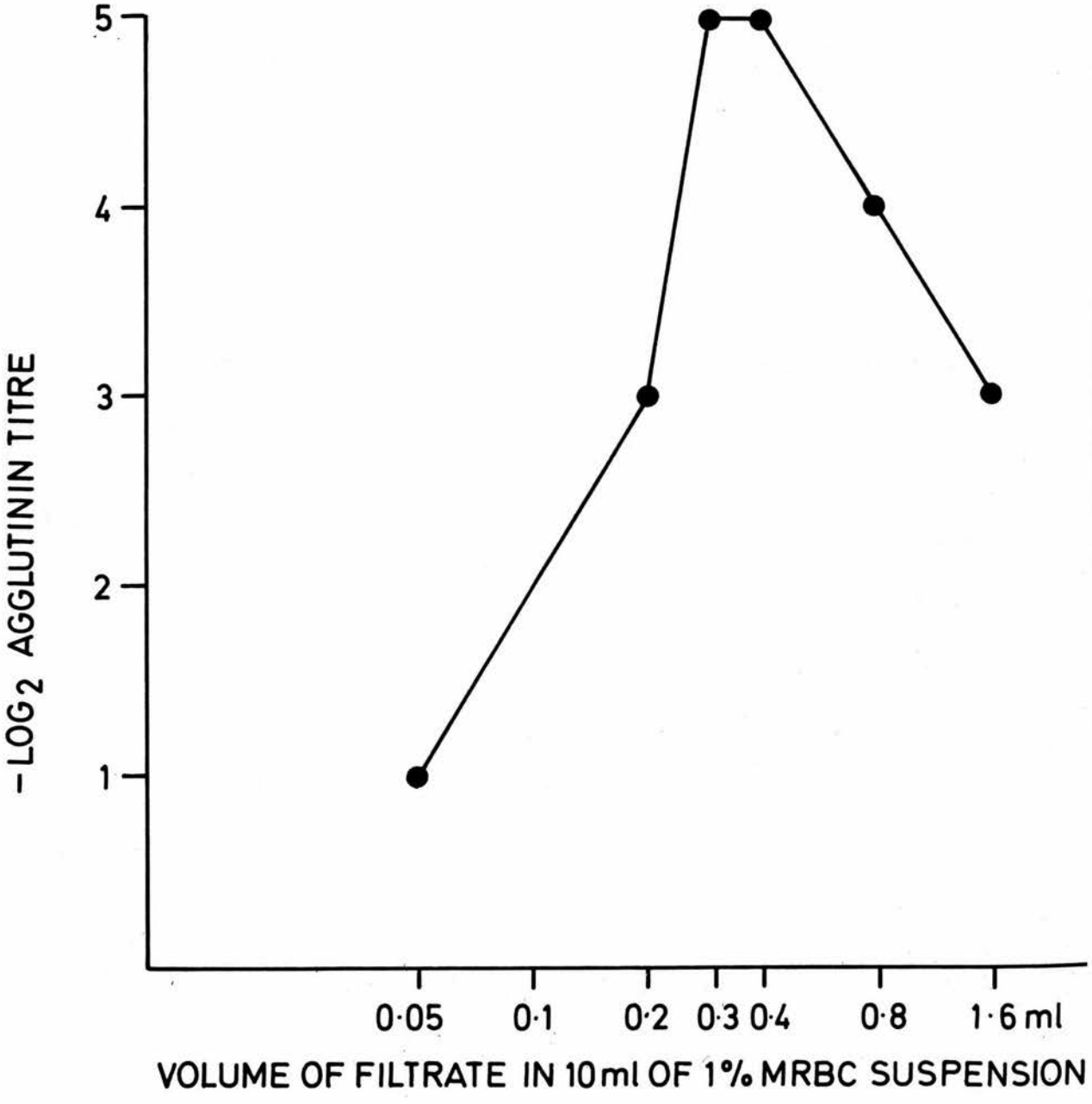


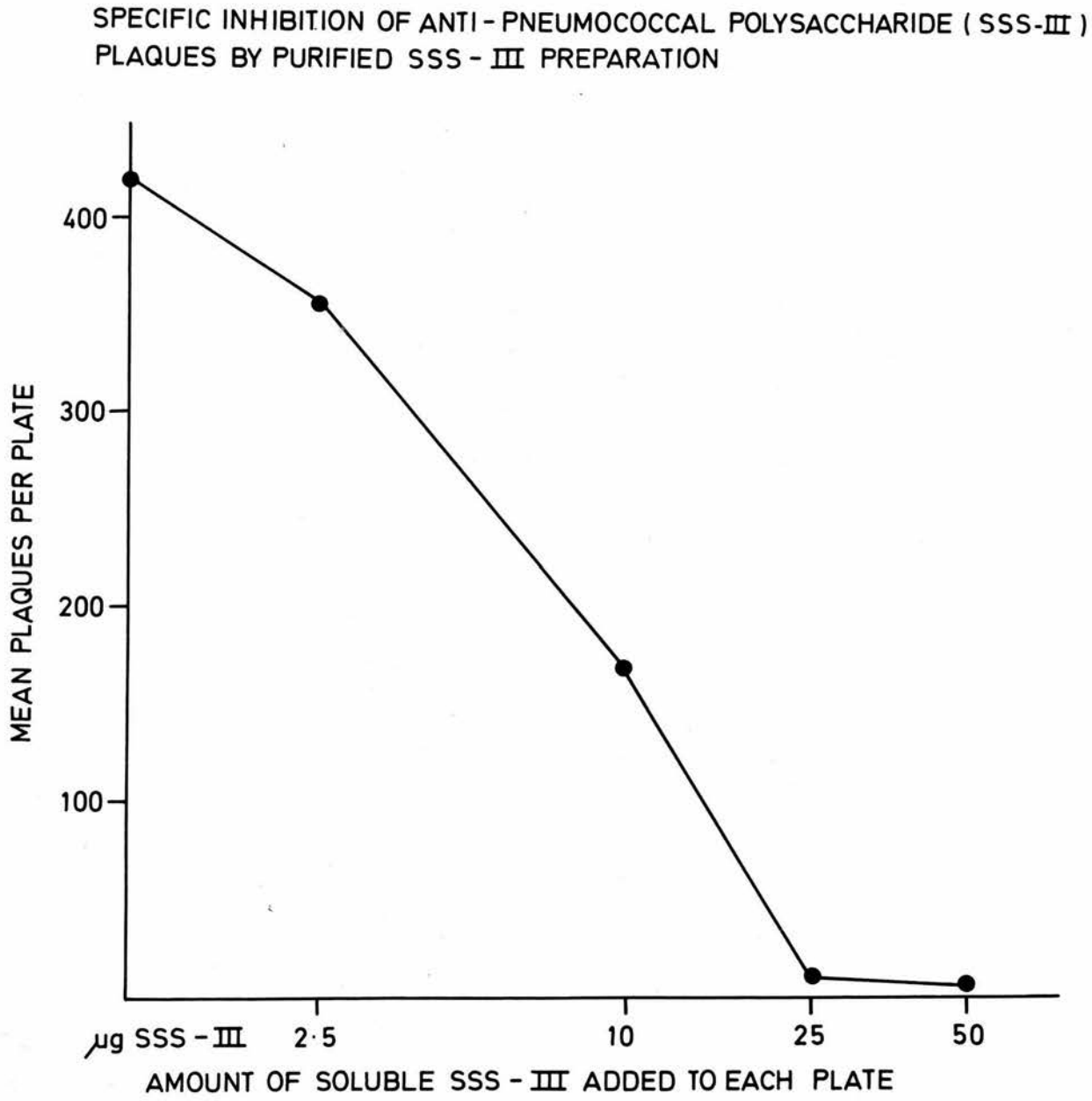

FIGURE 7

anti-pneumococcus polysaccharide plaques by the incorporation of purified type III pneumococcal polysaccharide. The results of this inhibition have been shown in Figure 8.

Haemagglutination assay: Mouse erythrocytes were obtained from freshly collected mouse blood. The cells were washed four times with PBS and made up to a 10 per cent (v/v) suspension. One ml. of this suspension was added to an optimum quantity of crude pneumococcus culture filtrate in 9 ml. of PBS. The suspension was incubated at 37°C for one hour with intermittent shaking. Finally the cells were washed four times with PBS and suspended at a concentration of 0.5 per cent in PBS containing 50 mg. BSA per 100 ml.

Serial doubling dilutions of the serum under test were made in 0.025 ml. PBS-BSA in V-shaped microtitre trays. An equal volume of 0.5 per cent (v/v) sensitized mouse erythrocytes was then added to each well and the plates were left at room temperature for two hours. They were then read macroscopically after tilting the plates. A tight button from which the cells did not slide down on tilting was considered a positive agglutination. For each group of sera only isologous mouse erythrocytes were used and the specificity of the test was ascertained by the inclusion of a standard rabbit anti-SSS-III serum (Difco) and a normal mouse serum with each test.

FIGURE 8


SPECIFIC INHIBITION OF ANTI-PNEUMOCOCCAL POLYSACCHARIDE (SSS-III) PLAQUES BY PURIFIED SSS-III PREPARATION
MEAN PLAQUES PER PLATE
400
300
200
100
μg SSS-III
2·5
10
25
50
AMOUNT OF SOLUBLE SSS-III ADDED TO EACH PLATE

Cell reconstitution of ALG-treated mice: The suspension of spleen and thymus cells used in these studies were prepared as described earlier. The bone marrow cells were obtained by infusing the femur with Eagle's medium and resuspending the cells by passing through a 21 gauge hypodermic needle. The required number of cells were slowly injected into the tail vein in a volume of 0.25 ml.

Anti-bovine serum albumin response

The antibodies to BSA were measured in individual sera by the ammonium sulphate method of Farr (1958) using the modifications described by Pinckard, Weir and McBride (1967). This briefly involved the following procedure.

The sera to be tested were diluted 1/10 in borate buffer (see Appendix I) containing 1 per cent normal mouse serum. From this four or five doubling dilutions were prepared in the same buffer. The dilutions of the test serum were selected on the basis of anticipated magnitude of the immune response and an end-point was always ensured. Half a ml. of each dilution of the test serum was transferred into Kahn tubes (75 x 12 mm. rimless glass test tubes). Together with test serum 3 sets of quadruplicate controls were also set up, 2 sets containing 0.5 ml. of a 1/10 dilution of normal mouse serum while the 3rd set contained the diluent only. To all the test and control tubes was added 0.5 ml. of the radio-iodinated BSA containing

0.04 or 0.4µg. protein nitrogen per ml. The tubes were incubated at 4°C overnight. The following morning all the tubes (except four of the controls containing normal serum) received 1 ml. of saturated ammonium sulphated solution. To the remaining four of the control tubes was added 1 ml. 20 per cent (w/v) trichloro-acetic acid (TCA). Immediately following the contents of the tubes were thoroughly mixed. The tubes were incubated at 4°C for 30 minutes and then spun at 2250g for 30 minutes in an MSE-Major centrifuge. The supernatants were discarded and the precipitates were resuspended in 3 ml. of saturated ammonium sulphate, or 20 per cent TCA as appropriate. The tubes were again incubated at 4°C for half an hour before recentrifugation. The precipitates were counted in a scintillation spectrometer (Nuclear Enterprise Mk II gamma-matic).

The two antigen concentrations, i.e. 0.04 and 0.4µg. protein nitrogen per ml. were used in order to determine the relative binding affinity of the antibody in the anti-sera.

The actual 33 per cent antigen binding capacities (ABC) were determined by computer analysis and the results are expressed as µg. of BSA nitrogen bound by one ml. of undiluted serum. The computer programme was kindly provided by Dr. W.M. McBride (Department of Bacteriology, Edinburgh University) and the analyses were carried out

by the Edinburgh Regional Computer Centre.

$^{125}$Iodine labelling of BSA: Five mg. of BSA (Armour) in 0.5 ml. PBS was added to 0.5 ml. PBS in a small beaker containing 2-4µci of carrier-free $^{125}$Iodine (Radiochemical Centre, Amersham, Bucks, England). The mixture was stirred with the aid of a magnetic device and while mixing 0.2 ml. of stock solution containing 0.05 per cent (w/v) chloramine-T (BDH, Poole, Dorset, England) in distilled water was added. The mixture was allowed to stand for five minutes before 3 ml. of borate buffer was added. After this the contents were carefully transferred into a dialysis tubing and dialysed overnight against a litre of borate buffer containing 10 per cent (w/v) potassium iodide. This was followed by a further dialysis for 72 hours against iodide-free borate buffer with 3-4 changes of the buffer. The protein content of the iodinated BSA preparation was determined by the micro-Kjeldahl procedure (Kabat and Mayer, 1961). Aliquots of the preparation were stored at -20°C and just before use they were diluted to the required protein concentration with borate buffer containing 1 per cent (v/v) normal mouse serum.

## PRESENTATION OF RESULTS AND STATISTICAL ANALYSIS

### Anti-sheep erythrocyte response

The arithmetic mean of the plaques on the two duplicate plates was obtained and the PFC content in the spleen of individual mice was calculated. Also the number of PFC per $10^6$ nucleated cells from individual spleens were calculated. From the developed plaques per spleen the number of corrected indirect plaques per spleen and those per $10^6$ nucleated spleen cells were determined. A geometric mean of the number of plaques per spleen or plaques per $10^6$ nucleated spleen cells was obtained for each experimental group. The results from each group have been expressed as the mean PFC with the limits of one standard error from the mean.

The arithmetic mean of both total and reduced reciprocal $\log_2$ haemagglutinin and haemolysin titres were calculated for each experimental group. The results for each group have been expressed as the mean $\log_2$ titre with the limits of one standard error from the mean.

### Anti-SSS-III response

The average number of plaques in two duplicate plates with unsensitized erythrocytes was deducted from the average number of plaques obtained from the two plates containing

sensitized cells. Thus the number of specific plaques per spleen and per $10^6$ nucleated spleen cells was calculated for each individual mouse. A geometric mean of the specific plaques per spleen and per $10^6$ nucleated cells was determined for each experimental group. The results have been expressed as the mean PFC with the limits of one standard error from the mean.

The agglutination results for each group have been expressed as the arithmetic mean of the $\log_2$ titres with the limits of one standard error from the mean.

Anti-BSA response

The anti-BSA responses have been expressed as the geometric mean values of antigen binding capacities of each mouse serum together with the limits of one standard error from the geometric mean. The relative binding affinities for individual sera were calculated using the following formula and have been expressed as the arithmetic mean with the limits of one standard error from the mean.

$$\text{Relative binding affinity} = \frac{\text{ABC with 0.02}\mu\text{g. protein N}}{\text{ABC with 0.2}\mu\text{g. protein N}} \times 100$$

Statistical analysis

Various experimental groups were compared by means of the two tail student's "t" test formula and the significance of the difference between any two groups has been expressed

as the "p" value. All the "p" values higher than 0.05 were regarded as 'not significant'.

# RESULTS

# RESULTS

## IMMUNE RESPONSE TO SHEEP ERYTHROCYTES

### Effect of ALG on the primary immune response in various mouse strains

Groups containing between 3 and 6 mice received 5 mg. NHIgG or ALG(ld) intraperitoneally on days -4 and -2 and were challenged with 3 x $10^8$ SRBC by intraperitoneal injection on day 0. The direct PFC response of the spleen and serum antibody titres were measured five days after the challenge. The results of these experiments have been summarized in Table 5.

It is quite obvious from the data that this ALG preparation has successfully suppressed the primary immune response to this dose of SRBC in all mouse strains tested. The magnitude of the response does not seem to determine the degree of suppression by the ALG treatment. For example, in A/HeJ mice, although the magnitude of the response is greater than any other strain to this dose of SRBC, the degree of suppression by ALG treatment is as good or even better than other strains which did not respond so well (e.g. C57Bl). In all instances the differences between the NHIgG- and ALG-treated groups were highly significant.

TABLE 5 - THE EFFECT OF ALG ON THE PRIMARY IMMUNE RESPONSE OF DIFFERENT MOUSE STRAINS AGAINST SHEEP ERYTHROCYTES[a]

| Mouse Strain | Direct (IgM) Plaque Forming Cells Per Spleen[b] | | P[c] |
|---|---|---|---|
| | NHIgG | ALG | |
| A/HeJ | 63,153 (57,347-69,546) (6)[d] | 2,167 (1,668-2,815) (6) | <0.001 |
| $C_{57}Bl$ | 40,274 (32,935-49,248) (4) | 2,282 (1,916-2,717) (4) | <0.001 |
| Balb/c | 54,170 (40,973-71,618) (5) | 9,733 (7,563-12,526) (5) | <0.01 |
| DBA/1 | 52,453 (46,878-58,690) (5) | 7,351 (6,324-8,544) (5) | <0.001 |
| CBA[e] | 20,157 (14,657-27,721) (4) | 1,336 (1,135-1,573) (4) | <0.001 |
| | 17,873 (15,586-20,496) (4) | 221 (142-343) (4) | <0.001 |
| $C_3H$[e] | 24,298 (22,272-26,507) (4) | 933 (519-1,677) (4) | <0.005 |
| | 33,738 (23,416-48,611) (3) | 2,416 (1,801-3,239) (3) | <0.005 |

(a) 5.0 mg. NHIgG or ALG injected i.p. on days -4 and -2; 3 x $10^8$ SRBC given i.p. on day 0 and tested on day 5.

(b) Geometric mean with the limits of one standard error.

(c) Comparison between NHIgG and ALG groups.

(d) Number of mice in each group.

(e) Observations from two different experiments.

In another set of experiments groups of mice were treated with NHIgG or ALG and challenged with SRBC as above. Four days after the antigenic challenge, the spleens were assayed for both IgM (direct) and IgG (developed) PFC. Although the size of the IgM responses in these experiments were lower (Table 6) in all mouse strains (except Balb/c) as compared with the responses on day 5 (Table 5), the ALG-treated groups invariably showed a severe depression in their responsiveness. In all cases the suppression was highly significant and in the majority of cases, the degree of suppression was greater than that observed when mice were tested on day 5 (compare Tables 5 and 6). The IgG response in these mice was weak, as would be expected at this time after the antigenic challenge (Table 7). Nevertheless, in all strains where there was a measurable IgG response in control groups, it was significantly suppressed by ALG treatment (note C57Bl, DBA/1 and C3H). However, in one instance (CBA) the ALG treatment caused a small but statistically significant potentiation of the primary IgG response. The significance of this potentiation will be discussed later.

The serological tests performed on sera from the above animals produced results which in general were consistent with those obtained by the PFC assay. From the results of haemagglutinating and haemolytic activities in sera obtained on days 4 and 5 after antigenic challenge it is

TABLE 6 - THE EFFECT OF ALG ON THE PRIMARY IgM IMMUNE RESPONSE OF DIFFERENT MOUSE STRAINS AGAINST SHEEP ERYTHROCYTES[a]

| Mouse Strain | Direct (IgM) Plaque Forming Cells Per Spleen[b] | | p[c] |
|---|---|---|---|
| | NHIgG | ALG | |
| A/HeJ | 12,745 (11,868-13,687) (5)[d] | 1,482 (1,168-1,880) (6) | <0.001 |
| $C_{57}Bl$ | 3,172 (1,955-5,146) (5) | 206 (118-360) (5) | <0.005 |
| Balb/c | 59,535 (52,119-68,005) (5) | 594 (469-752) (4) | <<0.001 |
| DBA/1 | 34,659 (27,939-42,996) (6) | 305 (236-393) (6) | <<0.001 |
| CBA | 26,739 (19,090-37,451) (5) | 208 (178-242) (5) | <<0.001 |
| $C_3H$ | 20,708 (17,946-23,894) (6) | 468 (386-566) (7) | <<0.001 |

(a) 5.0 mg. NHIgG or ALG injected i.p. on days -4 and -2; $3 \times 10^8$ SRBC given i.p. on day 0 and tested on day 4.

(b) Geometric mean with the limits of one standard error.

(c) Comparison between NHIgG and ALG groups.

(d) Number of mice in each group.

TABLE 7 - THE EFFECT OF ALG ON THE PRIMARY IgG RESPONSE OF DIFFERENT MOUSE STRAINS AGAINST SHEEP ERYTHROCYTES[a]

| Mouse Strain | Developed (IgG) Plaque Forming Cells Per Spleen[b] | | P[c] |
|---|---|---|---|
| | NHIgG | ALG | |
| A/HeJ | 233 (132-410) (5)[d] | 255 (133-490) (6) | N.S. |
| $C_{57}Bl$ | 3,435 (2,087-5,652) (5) | 54 (44-65) (5) | <0.001 |
| Balb/c | Neg | Neg | N.S. |
| DBA/1 | 14,783 (12,075-18,099) (6) | 170 (100-292) (6) | <0.001 |
| CBA | 23 (21-25) (5) | 124 (84-184) (5) | <0.005 |
| $C_3H$ | 1,294 (636-2,632) (6) | 68 (52-89) (7) | <0.005 |

(a) 5.0 mg. NHIgG or ALG injected i.p. on days -4 and -2; $3 \times 10^8$ SRBC given i.p. on day 0 and tested on day 4.

(b) Geometric mean with the limits of one standard error.

(c) Comparison between NHIgG and ALG groups: P values greater than 0.05 were considered not significant (NS.).

(d) Number of mice in each group.

apparent that 2-mercaptoethanol treatment drastically reduced the antibody titres in all cases. This was more noticeable in sera obtained on day 4 than in those obtained on day 5 (Tables 8-11). Moreover, ALG treatment in all instances caused significant reductions in both haemagglutinin and haemolysin responses. It is noteworthy that none of the strains produced any significant amount of 2-mercaptoethanol-resistant antibody following ALG treatment. In those cases where the suppression of 2-mercaptoethanol-resistant antibody was not statistically significant, it was obviously due to very poor response in the control groups.

## Effect of varying the antigen dose on the immunosuppressive ability of ALG

Groups of mice containing 3-8 mice were tested with 5 mg. of NHIgG or ALG intraperitoneally on days -4 and -2 and challenged with either $3 \times 10^7$ or $3 \times 10^9$ SRBC by the same route on day 0. Five days later they were tested for splenic IgM PFC responses and serum antibody titres. The results of PFC assays on these mice have been summarized in Table 12.

In all instances the control groups (NHIgG treated) challenged with $3 \times 10^7$ SRBC gave a poorer response than those challenged with $3 \times 10^9$ SRBC. When compared with the response of mice challenged with $3 \times 10^8$ SRBC, it appears that in all strains except Balb/c, $3 \times 10^7$ SRBC

TABLE 8 - THE EFFECT OF ALG ON THE PRIMARY HAEMAGGLUTININ RESPONSE OF DIFFERENT MOUSE STRAINS AGAINST SHEEP ERYTHROCYTES[a]

| Mouse Strain | 2-ME[e] Treatment | NHIgG | | ALG | | P[c] |
|---|---|---|---|---|---|---|
| | | No. of Mice | $Log_2$ Titre[b] | No. of Mice | $Log_2$ Titre | |
| A/HeJ | - | 6 | 5.83 ± 0.17 | 6 | 1.50 ± 0.22 | <0.001 |
| | + | | 2.50 ± 0.56 | | neg | <0.005 |
| $C_{57}Bl$ | - | 7 | 3.57 ± 0.37 | 7 | 1.86 ± 0.55 | <0.025 |
| | + | | 1.00 ± 0.38 | | 0.57 ± 0.30 | N.S. |
| Balb/c | - | 5 | 7.20 ± 0.37 | 5 | 1.60 ± 0.40 | <0.001 |
| | + | | 1.40 ± 0.40 | | neg | <0.01 |
| DBA/1 | - | 5 | 7.60 ± 0.24 | 5 | 1.80 ± 0.37 | <0.001 |
| | + | | 2.00 ± 0.55 | | neg | <0.01 |
| CBA[d] | - | 4 | 7.25 ± 0.63 | 4 | 1.25 ± 0.25 | <0.001 |
| | + | | 2.50 ± 0.64 | | neg | <0.005 |
| | - | 4 | 7.75 ± 0.48 | 4 | neg | <<0.001 |
| | + | | 3.25 ± 1.11 | | neg | <0.05 |
| $C_3H$[d] | - | 4 | 6.50 ± 0.29 | 4 | 1.50 ± 0.64 | <0.001 |
| | + | | 4.50 ± 0.50 | | neg | <0.001 |
| | - | 3 | 5.00 ± 0.00 | 3 | 1.00 ± 0.58 | <0.005 |
| | + | | 2.67 ± 1.33 | | neg | N.S. |

(a) 5.0 mg. NHIgG or ALG injected i.p. on days -4 and -2; $3 \times 10^8$ SRBC given i.p. on day 0 and tested on day 5.

(b) Arithmetic mean ± 1 standard error.

(c) Comparison between NHIgG and ALG groups: P values greater than 0.05 were considered not significant (N.S.).

(d) Observations from two different experiments.

(e) 2-Mercaptoethanol treatment.

TABLE 9 - THE EFFECT OF ALG ON THE PRIMARY HAEMOLYSIN RESPONSE OF DIFFERENT MOUSE STRAINS AGAINST SHEEP ERYTHROCYTES[a]

| Mouse Strain | 2-ME[e] Treatment | NHIgG | | ALG | | P[c] |
|---|---|---|---|---|---|---|
| | | No. of Mice | $Log_2$ Titre[b] | No. of Mice | $Log_2$ Titre | |
| A/HeJ | - | 6 | 6.50 ± 0.22 | 6 | 0.50 ± 0.34 | <0.001 |
| | + | | 2.17 ± 0.48 | | neg | <0.005 |
| $C_{57}Bl$ | - | 7 | 5.71 ± 0.52 | 7 | 0.71 ± 0.36 | <0.005 |
| | + | | neg | | neg | N.S. |
| Balb/c | - | 5 | 8.00 ± 0.55 | 5 | neg | <0.001 |
| | + | | 1.80 ± 0.73 | | neg | <0.05 |
| DBA/1 | - | 5 | 8.00 ± 0.45 | 5 | 0.20 ± 0.20 | <0.001 |
| | + | | 3.40 ± 1.03 | | neg | <0.02 |
| CBA[d] | - | 4 | 6.50 ± 0.29 | 4 | neg | <0.001 |
| | + | | 1.00 ± 1.00 | | neg | N.S. |
| | - | 4 | 9.25 ± 0.48 | 4 | neg | <<0.001 |
| | + | | 3.25 ± 0.25 | | neg | <<0.001 |
| $C_3H$[d] | - | 4 | 8.00 ± 0.00 | 4 | 0.75 ± 0.75 | <0.001 |
| | + | | 4.25 ± 0.75 | | neg | <0.001 |
| | - | 3 | 6.67 ± 0.33 | 3 | 1.00 ± 0.00 | <<0.001 |
| | + | | neg | | neg | N.S. |

(a) 5.0 mg. NHIgG ALG injected i.p. on days -4 and -2; $3 \times 10^8$ SRBC given i.p. on day 0 and tested on day 5.

(b) Arithmetic mean ± 1 standard error.

(c) Comparison between NHIgG and ALG groups: P values greater than 0.05 were considered not significant (N.S.).

(d) Observations from two different experiments.

(e) 2-Mercaptoethanol treatment.

TABLE 10 - THE EFFECT OF ALG ON THE PRIMARY HAEMAGGLUTININ RESPONSE OF DIFFERENT MOUSE STRAINS AGAINST SHEEP ERYTHROCYTES[a]

| Mouse Strain | 2-ME[e] Treatment | NHIgG | | ALG | | P[c] |
|---|---|---|---|---|---|---|
| | | No. of Mice | $Log_2$ Titre[b] | No. of Mice | $Log_2$ Titre | |
| A/HeJ | - | 5 | 6.60 ± 0.24 | 5 | 2.80 ± 0.37 | <<0.001 |
| | + | | 2.20 ± 0.20 | | 1.20 ± 0.58 | N.S. |
| $C_{57}Bl$ | - | 5 | 3.00 ± 0.32 | 5 | 0.40 ± 0.24 | <0.001 |
| | + | | neg | | neg | N.S. |
| Balb/c | - | 5 | 6.80 ± 0.20 | 4 | 1.50 ± 0.29 | <<0.001 |
| | + | | 1.20 ± 0.20 | | neg | <0.005 |
| DBA/1 | - | 6 | 6.83 ± 0.31 | 6 | 1.33 ± 0.82 | <<0.001 |
| | + | | neg | | neg | N.S. |
| CBA | - | 5 | 6.60 ± 0.40 | 5 | 1.60 ± 0.24 | <0.001 |
| | + | | neg | | neg | N.S. |
| $C_3H$[d] | - | 6 | 6.00 ± 0.68 | 6 | 2.33 ± 0.67 | <0.005 |
| | + | | 1.00 ± 0.45 | | neg | <0.05 |
| | - | 4 | 5.25 ± 0.25 | 4 | 0.25 ± 0.25 | <<0.001 |
| | + | | neg | | neg | N.S. |

(a) 5.0 mg. NHIgG or ALG injected i.p. on days -4 and -2; $3 \times 10^8$ SRBC given i.p. on day 0 and tested on day 4.

(b) Arithmetic mean ± 1 standard error.

(c) Comparison between NHIgG and ALG groups: P values greater than 0.05 were considered not significant (N.S.).

(d) Observations from two different experiments.

(e) 2-Mercaptoethanol treatment.

TABLE 11 - THE EFFECT OF ALG ON THE PRIMARY HAEMOLYSIN RESPONSE OF DIFFERENT MOUSE STRAINS AGAINST SHEEP ERYTHROCYTES[a]

| Mouse Strain | 2-ME[e] Treatment | NHIgG | | ALG | | P[c] |
|---|---|---|---|---|---|---|
| | | No. of Mice | $Log_2$ Titre[b] | No. of Mice | $Log_2$ Titre | |
| A/HeJ | - | 5 | 6.40 ± 0.24 | 5 | 1.00 ± 0.63 | <0.001 |
| | + | | neg | | neg | N.S. |
| $C_{57}Bl$ | - | 5 | 4.80 ± 0.49 | 5 | neg | <0.001 |
| | + | | neg | | neg | N.S. |
| Balb/c | - | 5 | 8.80 ± 0.20 | 5 | neg | <<0.001 |
| | + | | neg | | neg | N.S. |
| DBA/1 | - | 6 | 9.17 ± 0.31 | 6 | 0.67 ± 0.21 | <<0.001 |
| | + | | neg | | neg | N.S. |
| CBA | - | 5 | 8.20 ± 0.20 | 5 | neg | <<0.001 |
| | + | | neg | | neg | N.S. |
| $C_3H$[d] | - | 6 | 5.50 ± 0.99 | 6 | 1.00 ± 0.52 | <0.005 |
| | + | | neg | | neg | N.S. |
| | - | 4 | 6.25 ± 0.25 | 4 | 0.50 ± 0.29 | <<0.001 |
| | + | | neg | | neg | N.S. |

(a) 5.0 mg. NHIgG or ALG injected i.p. on days -4 and -2; $3 \times 10^8$ SRBC given i.p. on day 0 and tested on day 4.

(b) Arithmetic mean ± 1 standard error.

(c) Comparison between NHIgG and ALG groups: P values greater than 0.05 were considered not significant (N.S.).

(d) Observations from two different experiments.

(e) 2-Mercaptoethanol treatment.

are below the optimum dose and $3 \times 10^9$ are above it (compare Tables 5 and 6 with Table 12). It is also apparent from the results in Table 12 that different strains varied widely in their responsiveness to this antigen and the variability was far more obvious in groups challenged with the lower antigen dose, i.e. $3 \times 10^7$ SRBC. However, not much significance could be attached to these inter-strain variations at this stage, since all the strains were not tested at the same time. Also, the NHIgG pre-treatment of mice might have modified their responsiveness differently in different strains. Nevertheless, results of better controlled experiments which will be described in another section suggested that these interstrain variations were genuine. Thus A/HeJ and C57Bl mice responded very poorly whereas Balb/c and DBA/1 gave very high responses (see Appendix II).

From a comparison of NHIgG- and ALG-treated groups it is evident that ALG treatment effectively suppressed the immune response of all the strains to both doses of the antigen, although the degree of suppression generally was greater in mice receiving the lower dose of the antigen (Table 12).

The results of serological tests on these animals have been summarized in Tables 13 and 14. An increase in the amount of antigen resulted in an increase in both haemagglutinin and haemolysin titres in all strains of

TABLE 12 - THE EFFECT OF ANTIGEN DOSE ON THE SUPPRESSION OF PRIMARY IMMUNE RESPONSE AGAINST SHEEP ERYTHROCYTES BY ALG IN DIFFERENT MOUSE STRAINS[a]

| Mouse Strain | Ag Dose ($3x10^n$) | Direct (IgM) Plaque Forming Cells Per Spleen[b] | | P[c] |
|---|---|---|---|---|
| | | NHIgG | ALG | |
| A/HeJ | 7 | 8,734(4,984-15,305) (6)[d] | 278(209-370) (8) | <0.001 |
| | 9 | 43,686(37,027-51,543) (6) | 2,502(1,944-3,219) (7) | <<0.001 |
| $C_{57}Bl$ | 7 | 3,696(2,292-5,960) (3) | 1,271(1,019-1,585) (4) | N.S. |
| | 9 | 43,584(39,039-48,659) (4) | 3,842(3,206-4,605) (4) | <0.001 |
| Balb/c | 7 | 58,990(53,939-64,515) (5) | 273(230-323) (5) | <<0.001 |
| | 9 | 80,357(70,279-91,881) (5) | 13,097(10,455-16,407) (4) | <0.001 |
| DBA/1 | 7 | 22,185(19,377-25,400) (4) | 127(114-140) (5) | <<0.001 |
| | 9 | 36,799(32,489-41,681) (4) | 3,225(1,966-5,293) (5) | <0.005 |
| CBA | 7 | 9,893(5,853-16,720) (4) | 219(152-316) (5) | <0.001 |
| | 9 | 45,201(38,877-52,554) (3) | 4,767(3,688-6,162) (5) | <0.001 |
| $C_3H$[e] | 7 | 17,673(14,769-21,149) (4) | 236(212-262) (5) | <<0.001 |
| | | 19,151(17,273-21,233) (3) | 2,136(1,916-2,381) (3) | <0.001 |
| | 9 | 31,870(29,326-34,635) (4) | 3,615(2,819-4,636) (4) | <0.001 |
| | | 35,698(26,507-48,074) (3) | 10,644(8,345-13,576) (4) | <0.005 |

(a) 5.0 mg. NHIgG or ALG injected i.p. on days -4 and -2; $3 \times 10^7$ or $3 \times 10^9$ SRBC given i.p. on day 0 and tested on day 5.

(b) Geometric mean with the limits of one standard error.

(c) Comparison between NHIgG and ALG groups: P values greater than 0.05 were considered not significant (N.S.).

(d) Number of mice in each group.

(e) Observations from two different experiments.

TABLE 13 - THE EFFECT OF ANTIGEN DOSE ON THE SUPPRESSION OF PRIMARY IMMUNE RESPONSE AGAINST SHEEP ERYTHROCYTES BY ALG IN DIFFERENT MOUSE STRAINS[a]

| Mouse Strain | Ag Dose ($3 \times 10^n$) | Total Haemagglutinin Titre[b] | | | | P[c] | 2-ME Reduced Haemagglutinin Titre[b] | | | | P[c] |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | NHIgG | | ALG | | | NHIgG | | ALG | | |
| | | No. of Mice | $Log_2$ Titre | No. of Mice | $Log_2$ Titre | | No. of Mice | $Log_2$ Titre | No. of Mice | $Log_2$ Titre | |
| A/HeJ | 7 | 6 | 3.50 ± 0.56 | 8 | 0.87 ± 0.29 | <0.001 | 6 | neg | 6 | neg | N.S. |
| | 9 | 6 | 7.17 ± 0.48 | 7 | 3.47 ± 0.37 | <0.001 | 6 | 4.00 ± 0.26 | 7 | neg | <<0.001 |
| $C_{57}Bl$ | 7 | 7 | 3.28 ± 0.52 | 7 | 0.86 ± 0.11 | <0.005 | 7 | 0.71 ± 0.42 | 7 | neg | N.S. |
| | 9 | 7 | 4.43 ± 0.57 | 7 | 1.86 ± 0.51 | <0.01 | 7 | 1.28 ± 0.18 | 7 | 0.14 ± 0.14 | <0.001 |
| Balb/c | 7 | 5 | 7.20 ± 0.20 | 5 | 1.20 ± 0.20 | <<0.001 | 5 | neg | 5 | neg | N.S. |
| | 9 | 5 | 9.80 ± 0.20 | 4 | 5.00 ± 0.71 | <0.001 | 5 | 8.20 ± 0.58 | 4 | 0.50 ± 0.50 | <0.001 |
| DBA/1 | 7 | 5 | 6.00 ± 0.32 | 5 | 0.80 ± 0.49 | <0.001 | 5 | 0.80 ± 0.37 | 5 | neg | N.S. |
| | 9 | 4 | 7.00 ± 0.00 | 5 | 2.40 ± 0.24 | <<0.001 | 4 | 6.00 ± 0.41 | 5 | neg | <<0.001 |
| CBA | 7 | 4 | 6.25 ± 0.75 | 5 | 1.00 ± 0.45 | <0.001 | 4 | neg | 5 | neg | N.S. |
| | 9 | 4 | 7.25 ± 1.11 | 5 | 2.60 ± 0.60 | <0.01 | 4 | 4.00 ± 1.22 | 5 | 0.20 ± 0.20 | <0.02 |
| $C_3H^d$ | 7 | 4 | 4.00 ± 0.41 | 5 | 0.40 ± 0.24 | <0.001 | 4 | 0.75 ± 0.75 | 5 | neg | N.S. |
| | | 3 | 5.33 ± 0.67 | 3 | 1.00 ± 0.58 | <0.01 | 3 | neg | 3 | neg | N.S. |
| | 9 | 4 | 6.50 ± 0.29 | 4 | 2.25 ± 0.48 | <0.001 | 4 | 5.00 ± 0.41 | 4 | neg | <0.001 |
| | | 3 | 6.33 ± 0.33 | 4 | 2.25 ± 0.25 | <0.001 | 3 | 4.33 ± 0.33 | 4 | neg | <<0.001 |

(a) 5.0 mg. NHIgG or ALG injected i.p. on days -4 and -2; $3 \times 10^7$ or $3 \times 10^9$ SRBC given i.p. on day 0 and tested on day 5.
(b) Arithmetic mean ± 1 standard error.
(c) Comparison between NHIgG and ALG groups: P values greater than 0.05 were considered not significant (N.S.).
(d) Observations from two different experiments.

TABLE 14 - THE EFFECT OF ANTIGEN DOSE ON THE SUPPRESSION OF PRIMARY IMMUNE RESPONSE AGAINST SHEEP ERYTHROCYTES BY ALG IN DIFFERENT MOUSE STRAINS[a]

| Mouse Strain | Ag Dose ($3x10^n$) | Total Haemolysin Titre[b] | | | | P[c] | 2-ME Reduced Haemolysin Titre[b] | | | | P[c] |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | NHIgG | | ALG | | | NHIgG | | ALG | | |
| | | No. of Mice | $Log_2$ Titre | No. of Mice | $Log_2$ Titre | | No. of Mice | $Log_2$ Titre | No. of Mice | $Log_2$ Titre | |
| A/HeJ | 7 | 6 | 4.83 ± 0.70 | 8 | 0.50 ± 0.20 | <0.001 | 6 | 2.33 ± 0.76 | 8 | neg | <0.005 |
| | 9 | 6 | 8.33 ± 0.33 | 7 | 3.57 ± 0.65 | <0.001 | 6 | 5.50 ± 0.22 | 7 | neg | <<0.001 |
| $C_{57}Bl$ | 7 | 7 | 4.67 ± 0.56 | 7 | 0.71 ± 0.36 | <0.001 | 7 | neg | 7 | neg | N.S. |
| | 9 | 7 | 6.00 ± 0.38 | 7 | 0.86 ± 0.34 | <0.01 | 7 | neg | 7 | neg | N.S. |
| Balb/c | 7 | 5 | 8.80 ± 0.49 | 5 | neg | <<0.001 | 5 | 2.20 ± 0.97 | 5 | neg | N.S. |
| | 9 | 5 | 10.20 ± 0.49 | 5 | 5.75 ± 0.48 | <0.001 | 5 | 9.60 ± 0.23 | 5 | neg | <<0.001 |
| DBA/1 | 7 | 5 | 6.80 ± 0.20 | 5 | neg | <<0.001 | 5 | neg | 5 | neg | N.S. |
| | 9 | 4 | 8.50 ± 0.29 | 5 | 1.80 ± 0.49 | <0.001 | 4 | 5.25 ± 0.25 | 5 | neg | <<0.001 |
| CBA | 7 | 4 | 6.50 ± 0.87 | 5 | neg | <0.001 | 4 | neg | 5 | neg | N.S. |
| | 9 | 4 | 5.75 ± 1.93 | 5 | neg | <0.02 | 4 | 1.50 ± 0.87 | 5 | neg | N.S. |
| $C_3H^d$ | 7 | 4 | 7.25 ± 0.48 | 5 | neg | <<0.001 | 4 | 1.75 ± 1.03 | 5 | neg | N.S. |
| | | 3 | 5.67 ± 0.67 | 3 | 0.67 ± 0.33 | <0.001 | 3 | neg | 3 | neg | N.S. |
| | 9 | 4 | 9.00 ± 0.41 | 4 | 1.00 ± 1.00 | <0.001 | 4 | 4.25 ± 0.25 | 4 | neg | <<0.001 |
| | | 3 | 7.33 ± 0.33 | 4 | 1.25 ± 0.25 | <<0.001 | 3 | neg | 4 | neg | N.S. |

(a) 5.0 mg. NHIgG or ALG injected i.p. on days -4 and -2; $3 \times 10^7$ or $3 \times 10^9$ SRBC given i.p. on day 0 and tested on day 5.
(b) Arithmetic mean ± 1 standard error.
(c) Comparison between NHIgG and ALG groups: P values greater than 0.05 were considered not significant (N.S.).
(d) Observations from two different experiments.

mice treated with NHIgG. Also, an increase in the amount of antigen resulted in higher titres of 2-mercaptoethanol-resistant haemagglutinin as well as haemolysins. Nevertheless the ALG pretreatment significantly suppressed the total haemagglutinin and haemolysin responses in all mouse strains challenged with both high and low antigen doses. In addition, no significant amount of 2-mercaptoethanol-resistant antibody was produced by ALG treated animals in any of the mouse strains tested.

Effect of ALG on the secondary immune response of different mouse strains

Groups comprising of 3-6 mice were sensitized with $3 \times 10^8$ SRBC and 3-4 weeks thereafter they received two doses of 5 mg. each of NHIgG or ALG(1d) two days apart. Two days following this treatment, mice were challenged with $3 \times 10^8$ SRBC and the PFC responses of spleens were determined four days later. The results of these experiments have been summarized in Tables 15 and 16.

Treatment with ALG effectively reduced the number of both IgM and IgG PFC (possibly some IgA plaques too) in all mouse strains, although the reductions were more pronounced in some strains (e.g. Balb/c and DBA/1) than in others (A/HeJ). The total haemagglutinin and haemolysin responses were also significantly suppressed by the ALG treatment in most mouse strains although the suppression

TABLE 15 - THE EFFECT OF ALG ON THE SECONDARY IgM IMMUNE RESPONSE OF DIFFERENT MOUSE STRAINS AGAINST SHEEP ERYTHROCYTES[a]

| Mouse Strain | Direct (IgM) Plaque Forming Cells Per Spleen[b] | | P[c] |
|---|---|---|---|
| | NHIgG | ALG | |
| A/HeJ[e] | 10,624 (5,891-19,157) (5)[d] | 2,902 (2,076-4,057) (6) | N.S. |
| | 18,506 (22,539-38,891 (5) | 1,711 (1,046-2,810) (5) | ≤0.005 |
| $C_{57}$Bl | 23,470 (19,082-28,868) (6) | 510 (355-734) (6) | ≤0.001 |
| Balb/c[e] | 12,219 (9,678-15,429) (6) | 1,046 (961-1,318) (6) | ≤≤0.001 |
| | 5,124 (3,090-8,497) (5) | 571 (436-748) (5) | ≤0.01 |
| DBA/1[e] | 10,006 (7,263-13,784) (6) | 138 (83-230) (6) | ≤0.001 |
| | 5,183 (4,254-6,315) (5) | 77 (64-92) (5) | ≤≤0.001 |
| CBA | 10,664 (9,817-11,573) (4) | 515 (340-781) (6) | ≤0.001 |
| $C_3H$[e] | 7,529 (5,167-10,972) (4) | 789 (247-499) (6) | ≤0.01 |
| | 3,647 (2,278-5,838) (3) | 457 (384-544) (4) | ≤0.01 |

(a) 5.0 mg. NHIgG or ALG injected i.p. on days -4 and -2; 3 x $10^8$ SRBC given i.p. on day 0 and tested on day 4 (mice were primed with 3 x $10^8$ SRBC 3-4 weeks before the ALG treatment).
(b) Geometric mean with the limits of one standard error.
(c) Comparison between NHIgG and ALG groups: P values greater than 0.05 were considered not significant (N.S.).
(d) Number of mice in each group.
(e) Observations from two different experiments.

TABLE 16 - THE EFFECT OF ALG ON THE SECONDARY IgG (AND IgA?) IMMUNE RESPONSES OF DIFFERENT MOUSE STRAINS AGAINST SHEEP ERYTHROCYTES[a]

| Mouse Strain | Developed Plaque Forming Cells Per Spleen[b] | | P[c] |
|---|---|---|---|
| | NHIgG | ALG | |
| A/HeJ[e] | 254,125 (179,329-360,117) (5)[d] | 28,757 (20,546-40,251) (6) | <0.005 |
| | 198,901 (153,801-257,227) (5) | 12,869 (8,732-18,966) (5) | <0.001 |
| $C_{57}Bl$ | 186,494 (149,511-232,624) (6) | 6,050 (4,002-9,045) (6) | <0.001 |
| Balb/c[e] | 228,996 (194,700-269,333) (6) | 2,215 (1,228-3,997) (6) | <0.001 |
| | 233,212 (202,551-268,514) (5) | 6,480 (5,545-7,572) (5) | <<0.001 |
| DBA/1[e] | 327,170 (265,595-403,021) (6) | 6,332 (4,318-9,286) (6) | <0.001 |
| | 239,551 (225,773-254,169) (5) | 1,728 (1,164-2,566) (6) | <<0.001 |
| CBA | 342,567 (296,015-396,439) (4) | 7,880 (6,300-9,856) (6) | <<0.001 |
| $C_3H$[e] | 173,007 (135,058-221,619) (4) | 4,160 (2,549-6,791) (4) | <0.001 |
| | 133,428 (57,315-310,616 (3) | 4,779 (3,985-5,731) (4) | <0.01 |

(a) 5.0 mg. NHIgG or ALG injected i.p. on days -4 and -2; $3 \times 10^8$ SRBC given i.p. on day 0 and tested on day 4 (mice were primed with $3 \times 10^8$ SRBC 3-4 weeks prior to ALG treatment).
(b) Geometric mean with the limits of one standard error.
(c) Comparison between NHIgG and ALG groups.
(d) Number of mice in each group.
(e) Observations from two different experiments.

of the secondary circulating antibodies was not as striking as the suppression of the primary responses (Tables 17 and 18). The only exception in these experiments were C3H mice in which the suppression of secondary haemagglutinin and haemolysin responses by ALG treatment were variable. Treatment of sera from these mice with 2-mercaptoethanol caused very little reduction in the haemagglutinin titres although the haemolysin titres were more susceptible to this treatment. Furthermore, ALG treatment generally was more effective in suppressing 2-mercaptoethanol-resistant antibody titres (IgG responses) than it was in suppressing the total antibody titres. Again, in C3H mice the suppression of 2-mercaptoethanol-resistant antibodies was variable.

In another series of experiments following the same schedule of ALG treatment and antigen administration as in the secondary responses described above, mice were tested for the splenic PFC producing antibodies of different immunoglobulin classes and subclasses. The results of these tests have been summarized in Table 19.

It is apparent that ALG(ld) suppressed the responses of Balb/c, DBA/1 and C3H mice in all antibody classes and subclasses. However, in Balb/c mice the suppression of $IgG_{2b}$ and IgA responses were not statistically significant. The latter may be due to a wide variation in the immune response of individual mice in these immunoglobulin classes.

TABLE 17 - THE EFFECT OF ALG ON THE SECONDARY HAEMAGGLUTININ RESPONSE OF DIFFERENT MOUSE STRAINS AGAINST SHEEP ERYTHROCYTES[a]

| Mouse Strain | 2-ME[e] Treatment | NHIgG | | ALG | | P[c] |
|---|---|---|---|---|---|---|
| | | No. of Mice | $Log_2$ Titre[b] | No. of Mice | $Log_2$ Titre | |
| A/HeJ[d] | - | 5 | 13.80 ± 0.58 | 7 | 9.57 ± 0.30 | <0.001 |
| | + | | 12.80 ± 0.58 | | 9.28 ± 0.47 | <0.005 |
| | - | 5 | 10.40 ± 0.40 | 5 | 8.40 ± 0.60 | <0.025 |
| | + | | 10.60 ± 0.24 | | 7.20 ± 0.37 | <0.001 |
| $C_{57}Bl$ | - | 6 | 8.50 ± 0.22 | 6 | 7.00 ± 0.45 | <0.02 |
| | + | | 8.33 ± 0.21 | | 6.67 ± 0.42 | <0.01 |
| Balb/c[d] | - | 6 | 10.67 ± 0.21 | 6 | 7.33 ± 0.21 | <<0.001 |
| | + | | 10.67 ± 0.21 | | 5.33 ± 0.21 | <<0.001 |
| | - | 5 | 11.20 ± 0.37 | 5 | 7.60 ± 0.51 | <0.001 |
| | + | | 11.20 ± 0.37 | | 7.60 ± 0.51 | <0.001 |
| DBA/1[d] | - | 6 | 10.50 ± 0.22 | 6 | 7.50 ± 0.72 | <0.005 |
| | + | | 7.83 ± 0.40 | | 3.67 ± 0.56 | <0.001 |
| | - | 5 | 10.40 ± 0.24 | 5 | 6.83 ± 0.65 | <0.005 |
| | + | | 8.40 ± 0.40 | | 4.33 ± 0.21 | <0.001 |
| CBA | - | 4 | 11.25 ± 0.75 | 6 | 8.00 ± 0.26 | <0.005 |
| | + | | 9.00 ± 0.00 | | 5.83 ± 0.60 | <0.005 |
| $C_3H$[d] | - | 6 | 11.33 ± 1.38 | 7 | 9.57 ± 0.37 | N.S. |
| | + | | 10.50 ± 1.38 | | 8.43 ± 0.92 | N.S. |
| | - | 4 | 10.00 ± 1.08 | 5 | 8.60 ± 0.87 | N.S. |
| | + | | 10.25 ± 0.87 | | 5.80 ± 0.49 | <0.001 |

(a) 5.0 mg. NHIgG or ALG injected i.p. on days -4 and -2; $3 \times 10^8$ SRBC given i.p. on day 0 and tested on day 4 (mice were primed with $3 \times 10^8$ SRBC 3-4 weeks prior to ALG treatment).
(b) Arithmetic mean ± 1 standard error.
(c) Comparison between NHIgG and ALG groups: P values greater than 0.05 were considered not significant (N.S.).
(d) Observations from two different experiments.
(e) 2-Mercaptoethanol treatment.

TABLE 18 - THE EFFECT OF ALG ON THE SECONDARY HAEMOLYSIN RESPONSE OF DIFFERENT MOUSE STRAINS AGAINST SHEEP ERYTHROCYTES[a]

| Mouse Strain | 2-ME[e] Treatment | NHIgG | | ALG | | P[c] |
|---|---|---|---|---|---|---|
| | | No. of Mice | $Log_2$ Titre[b] | No. of Mice | $Log_2$ Titre | |
| A/HeJ[d] | - | 5 | 8.80 ± 0.37 | 7 | 4.28 ± 0.28 | <<0.001 |
| | + | | 7.40 ± 0.24 | | 2.43 ± 0.57 | <0.001 |
| | - | 5 | 8.00 ± 0.32 | 5 | 5.60 ± 0.24 | <0.001 |
| | + | | 8.00 ± 0.32 | | 4.40 ± 0.24 | <0.001 |
| $C_{57}Bl$ | - | 6 | 9.17 ± 0.54 | 6 | 5.83 ± 0.31 | <0.001 |
| | + | | 7.00 ± 0.36 | | 3.67 ± 0.88 | <0.01 |
| Balb/c[d] | - | 6 | 11.40 ± 0.24 | 6 | 6.33 ± 0.21 | <<0.001 |
| | + | | 6.83 ± 0.31 | | 0.50 ± 0.22 | <<0.001 |
| | - | 5 | 9.40 ± 0.40 | 5 | 6.60 ± 0.24 | <0.001 |
| | + | | 8.40 ± 0.40 | | 4.80 ± 0.49 | <0.001 |
| DBA/1[d] | - | 6 | 8.33 ± 0.21 | 6 | 5.67 ± 0.56 | <0.005 |
| | + | | 6.33 ± 0.33 | | 1.33 ± 0.33 | <<0.001 |
| | - | 5 | 8.20 ± 0.20 | 5 | 4.67 ± 0.33 | <0.001 |
| | + | | 6.80 ± 0.20 | | 1.17 ± 0.17 | <<0.001 |
| CBA | - | 4 | 7.75 ± 0.25 | 4 | 4.83 ± 0.17 | <0.001 |
| | + | | 5.50 ± 0.29 | | 1.50 ± 0.22 | <0.001 |
| $C_3H$[d] | - | 6 | 6.67 ± 0.42 | 7 | 4.71 ± 0.28 | <0.005 |
| | + | | 3.83 ± 0.87 | | 2.14 ± 0.80 | N.S. |
| | - | 4 | 7.75 ± 0.48 | 5 | 5.20 ± 0.37 | <0.005 |
| | + | | 5.50 ± 0.29 | | 1.20 ± 0.20 | <<0.001 |

(a) 5.0 mg. NHIgG or ALG injected i.p. on days -4 and -2; $3 \times 10^8$ SRBC given i.p. on day 0 and tested on day 4 (mice were primed with $3 \times 10^8$ SRBC 3-4 weeks prior to ALG treatment).
(b) Arithmetic mean ± 1 standard error.
(c) Comparison between NHIgG and ALG groups: P values greater than 0.05 were considered not significant (N.S.).
(d) Observations from two different experiments.
(e) 2-Mercaptoethanol treatment.

In A/HeJ mice, ALG treatment resulted in a suppression of all antibody classes except $IgG_{2a}$, which was slightly enhanced. The significance of this enhancement will be discussed later (Table 19).

## Effect of different ALG preparations on the immune response of CBA mice

The effect of four ALG preparations from individual horses were studied in CBA mice. These included the preparation (1d) used in the bulk of these studies, while the other three preparations were raised in different horses using different schedules of immunization with thymocytes from Swiss albino mice (see Table 2).

Groups containing 4-5 animals were treated with 5 mg. of NHIgG or different ALG preparations on days -4 and -2 and were challenged on day 0 with $3 \times 10^8$ SRBC. Five days after the challenge the spleens from these mice were assayed for their PFC responses. In these experiments both IgM and IgG responses were measured.

The results of IgM (direct) and IgG (developed) PFC in the spleens of various groups of animals have been listed in Table 20. Although the effectiveness of individual ALG preparations to suppress IgM PFC responses varied, they all significantly suppressed this response. It should be noted that the IgG responses were more readily suppressed by the same ALG preparations than the IgM responses.

TABLE 19 - THE EFFECT OF ALG ON THE SECONDARY IMMUNE RESPONSE OF DIFFERENT MOUSE STRAINS IN DIFFERENT IMMUNOGLOBULIN CLASSES AND SUB-CLASSES[a]

| Mouse Strain | Ig Class of PFC | NHIgG | | ALG | | P[c] |
|---|---|---|---|---|---|---|
| | | No. of Mice | PFC Per Spleen[b] | No. of Mice | PFC Per Spleen[b] | |
| A/HeJ | IgM | 5 | 18,506 (12,539-38,891) | 5 | 1,711 (1,042-2,810) | <0.005 |
| | $IgG_1$ | 5 | 52,941 (34,190-81,974) | 5 | 5,794 (4,101-8,186) | <0.005 |
| | $IgG_{2a}$ | 5 | 15,645 (12,183-20,090) | 5 | 16,898 (15,303-18,660) | N.S. |
| | $IgG_{2b}$ | 5 | 45,540 (28,370-73,102) | 5 | 516 (205-1,297) | <0.005 |
| | IgA | 5 | 20,459 (10,442-40,088) | 5 | 1,685 (1,016-2,795) | <0.02 |
| Balb/c | IgM | 5 | 5,124 (3,090-8,497) | 5 | 571 (436-748) | <0.01 |
| | $IgG_1$ | 5 | 62,267 (37,977-102,977) | 5 | 1,172 (738-1,862) | <0.001 |
| | $IgG_{2a}$ | 5 | 31,654 (23,964-41,812) | 5 | 5,975 (5,253-6,795) | <0.001 |
| | $IgG_{2b}$ | 5 | 6,917 (2,567-18,638) | 5 | 1,863 (1,472-2,358) | N.S. |
| | IgA | 5 | 4,825 (1,583-14,706) | 5 | 1,200 (877-1,641) | N.S. |
| DBA/1 | IgM | 5 | 5,183 (4,254-6,315) | 6 | 77 (64-92) | <<0.001 |
| | $IgG_1$ | 5 | 124,215 (111,799-138,010) | 6 | 714 (492-1,034) | <<0.001 |
| | $IgG_{2a}$ | 5 | 59,878 (50,353-71,205) | 6 | 469 (413-532) | <<0.001 |
| | $IgG_{2b}$ | 5 | 65,733 (53,879-80,195) | 6 | 521 (335-810) | <0.001 |
| | IgA | 5 | 79,216 (68,451-91,675) | 6 | 413 (260-655) | <<0.001 |
| $C_3H$ | IgM | 4 | 7,529 (5,167-10,972) | 4 | 789 (247-499) | <0.01 |
| | $IgG_1$ | 4 | 55,156 (37,553-81,009) | 4 | 339 (279-411) | <0.001 |
| | $IgG_{2a}$ | 4 | 39,951 (29,930-53,326) | 4 | 1,567 (1,029-2,386) | <0.001 |
| | $IgG_{2b}$ | 4 | 34,292 (24,079-48,836) | 4 | 407 (253-654) | <0.001 |
| | IgA | 4 | 20,099 (12,556-32,174) | 4 | 484 (384-610) | <0.001 |

(a) 0.5 mg. NHIgG or ALG injected i.p. on day -4 and -2; $3 \times 10^8$ SRBC given i.p. on day 0 and tested on day 4 (mice were primed with $3 \times 10^8$ SRBC 3-4 weeks prior to ALG treatment).
(b) Geometric mean with the limits of one standard error.
(c) Comparison between NHIgG and ALG groups: P values greater than 0.05 were considered not significant (N.S.).

TABLE 20 - THE EFFECT OF VARIOUS ALG PREPARATIONS ON THE IMMUNE RESPONSE OF CBA MICE AGAINST SHEEP ERYTHROCYTES[a]

| ALG Prep. | Plaque Forming Cells Per Spleen[b] | |
|---|---|---|
| | IgM (direct) | IgG (developed) |
| NHIgG | 17,123(14,718-19,921) (4)[d] | 19,374(12,414-30,238) (4) |
| ALG1d | 222(158-312) (5) | 29(13-66) (5) |
| P[c] | <0.001 | <0.001 |
| ALG2 | 1,252(1,069-1,467) (3) | 110(70-173) (3) |
| P | <0.001 | <0.001 |
| ALG3 | 5,290(3,633-7,690) (5) | 2,136(1,110-4,113) (5) |
| P | <0.05 | <0.005 |
| ALG4 | 3,122(2,071-4,707) (5) | 1,306(709-2,408) (5) |
| P | <0.01 | <0.02 |

(a) 5.0 mg. NHIgG or ALG injected i.p. on days -4 and -2; 3 x $10^8$ SRBC given i.p. on day 0 and tested on day 5.

(b) Geometric mean with the limits of one standard error.

(c) Comparison between NHIgG and ALG groups.

(d) Number of mice in each group.

However, the preparations least effective in suppressing the IgM responses (e.g. ALG3) were likewise least effective in suppressing the IgG responses. Conversely, the preparations most effective at suppressing the IgM responses were also very effective at suppressing IgG responses (e.g. ALG 1d).

The results of serological tests on these sera closely followed the pattern observed in the PFC assays. Namely, the antiserum least effective at suppressing PFC responses was also relatively less effective at suppressing haemagglutinin and haemolysin responses (Table 21). Nevertheless, all the ALG preparations significantly suppressed the circulating antibody formation.

Effect of ALG prepared from successive bleeds from a single horse

In these experiments groups of CBA mice (4-5 animals per group) were pretreated with NHIgG or ALG prepared from different bleeds from a single horse at different stages of immunization (see Table 2). The dose and schedule of ALG administration and antigenic challenge were similar to those described above. The results of PFC assays on spleens from these mice 5 days after antigenic challenge, have been summarized in Table 22.

All ALG preparations except 1a (obtained on day 21 after a single injection of thymocytes) significantly

TABLE 21 - THE EFFECT OF VARIOUS ALG PREPARATIONS ON THE CIRCULATING ANTIBODY RESPONSE OF CBA MICE AGAINST SHEEP ERYTHROCYTES[a]

| ALG Prep. | Haemagglutinin[b] | | Haemolysins[b] | |
|---|---|---|---|---|
| | Total | Reduced[e] | Total | Reduced[e] |
| NHIgG | 9.00 ± 0.87 (4)[d] | 5.25 ± 0.85 (4) | 9.50 ± 0.87 (4) | 6.75 ± 0.63 (4) |
| ALG1d | neg (5) | neg (5) | neg (5) | neg (5) |
| P[c] | <0.001 | <0.001 | <<0.001 | <<0.001 |
| ALG2 | 1.00 ± 0.58 (3) | neg (3) | 1.67 ± 0.88 (3) | neg (3) |
| P | <0.005 | <0.005 | <0.005 | <0.001 |
| ALG3 | 3.00 ± 0.63 (5) | 1.20 ± 0.58 | 5.00 ± 0.89 (5) | 2.40 ± 0.81 (5) |
| P | <0.005 | <0.005 | <0.01 | <0.005 |
| ALG4 | 2.20 ± 0.58 (5) | 0.60 ± 0.40 (5) | 3.40 ± 0.93 (5) | 1.60 ± 0.81 (5) |
| P | <0.005 | <0.005 | <0.005 | <0.005 |

(a) 5.0 mg. NHIgG or ALG injected i.p. on days -4 and -2; $3 \times 10^8$ SRBC given i.p. on day 0 and tested on day 5.

(b) Arithmetic mean ± 1 standard error.

(c) Comparison between NHIgG and ALG groups.

(d) Number of mice in each group.

(e) 2-Mercaptoethanol reduced.

TABLE 22 - THE RELATIONSHIP BETWEEN THE ABILITY OF ALG TO SUPPRESS THE IMMUNE RESPONSE OF CBA MICE TO SHEEP ERYTHROCYTES AND THE HYPERIMMUNIZATION OF THE ALG DONOR[a]

| ALG Prep. | Plaque Forming Cells Per Spleen[b] | |
|---|---|---|
| | IgM (direct) | IgG (developed) |
| NHIgG | 17,873(15,586-20,496) (4)[d] | 16,566(14,549-18,863) (4) |
| ALG1a | 15,246(12,492-18,607) (5) | 5,207(4,103-6,608) (5) |
| P[c] | N.S. | <0.01 |
| ALG1b | 6,836(5,815-8,037) (5) | 905(445-1,838) (5) |
| P | <0.005 | <0.01 |
| ALG1c | 1,935(1,054-3,554) (5) | 208(178-243) (5) |
| P | <0.02 | <<0.001 |
| ALG1d | 221(142-343) (4) | 33(12-94) (4) |
| P | <<0.001 | <0.005 |

(a) 5.0 mg. NHIgG or ALG injected i.p. on days -4 and -2; $3 \times 10^8$ SRBC given i.p. on day 0 and tested on day 5.

(b) Geometric mean with the limits of one standard error.

(c) Comparison between NHIgG and ALG groups: P values greater than 0.05 were considered not significant (N.S.).

(d) Number of mice in each group.

suppressed the IgM response, although the degree of suppression varied from one preparation to the other. It should be noted that the immunosuppressive capacity of the ALG preparations increased with the number of immunizing injections of the donor horse. The differences between the immunosuppression caused by different ALG preparations was significant in most cases ( 1a<0.02 1b(n.s.) 1c<0.001 1d<0.001 1a ). From the data on Table 22 it is also apparent that IgG responses were relatively more susceptible to suppression by all ALG preparations than the IgM responses. It should be pointed out that ALG preparation 1a significantly suppressed the IgG PFC response but not the IgM response.

The serological data from the above experiments is recorded in Table 23. This data strengthens the findings obtained with the PFC assays.

Recovery of the immunocompetence after ALG treatment

Groups containing a minimum of four mice were treated with ALG(1d) on days -4 and -2 and challenged on day 0 with $3 \times 10^8$ SRBC. Thereafter the mice were bled at various intervals from the retro-orbital sinus. These mice were rechallenged at 13-17 weeks with $3 \times 10^8$ SRBC and exsanguinated 6 days later. All the sera were tested for haemagglutinating antibodies.

The results of one such experiment in CBA mice have been summarized in Figure 9. It is apparent that following

TABLE 23 - THE RELATIONSHIP BETWEEN THE ABILITY OF ALG TO SUPPRESS THE CIRCULATING ANTIBODY RESPONSE OF CBA MICE TO SHEEP ERYTHROCYTES AND THE HYPERIMMUNIZATION OF THE ALG DONOR[a]

| ALG Prep. | Haemagglutinin[b] | | Haemolysins[b] | |
|---|---|---|---|---|
| | Total | 2-ME-Reduced | Total | 2-ME-Reduced |
| NHIgG | 7.75 ± 0.48 (4)[d] | 3.25 ± 1.11 (4) | 9.25 ± 0.48 (4) | 3.25 ± 0.25 (4) |
| ALG1a | 6.80 ± 0.20 (5) | 2.00 ± 0.45 (5) | 8.80 ± 0.20 (5) | neg (5) |
| P[c] | <0.05 | N.S. | N.S. | <<0.001 |
| ALG1b | 4.40 ± 0.24 (5) | 0.40 ± 0.24 (5) | 6.40 ± 0.24 (5) | neg (5) |
| P | <0.001 | <0.05 | <0.001 | <<0.001 |
| ALG1c | 2.00 ± 0.55 (5) | neg (5) | 3.60 ± 0.93 (5) | neg (5) |
| P | <0.001 | <0.02 | <0.005 | <<0.001 |
| ALG1d | neg (4) | neg (4) | neg (4) | neg (4) |
| P | <<0.001 | <0.025 | <<0.001 | <<0.001 |

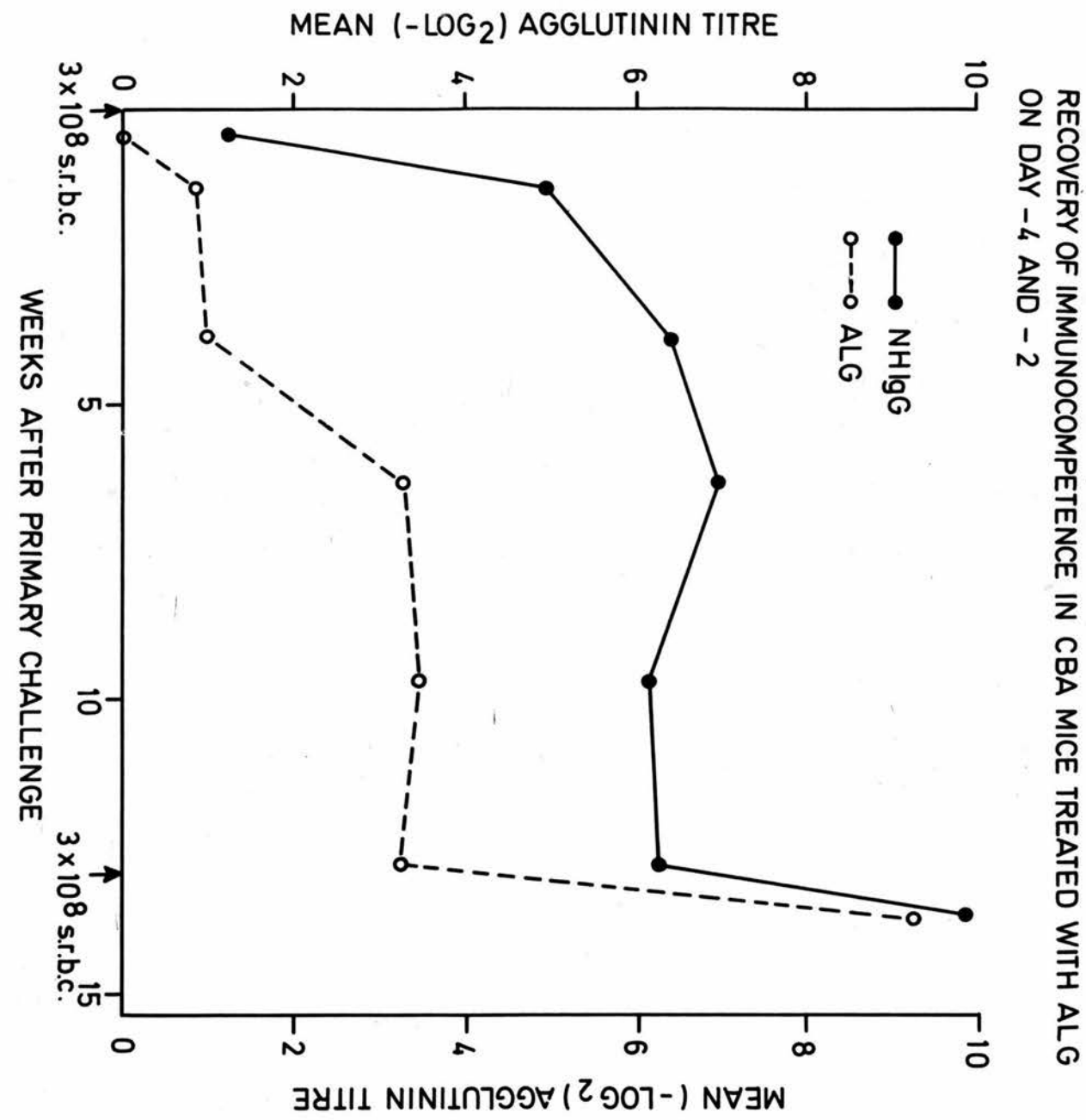
(a) 5.0 mg. NHIgG or ALG injected i.p. on days -4 and -2; $3 \times 10^8$ SRBC given i.p. on day 0 and tested on day 5.

(b) Arithmetic mean ± 1 standard error.

(c) Comparison between NHIgG and ALG groups: P values greater than 0.05 were considered not significant (N.S.).

(d) Number of mice in each group.

FIGURE 9


RECOVERY OF IMMUNOCOMPETENCE IN CBA MICE TREATED WITH ALG ON DAY -4 AND -2
MEAN (-LOG2) AGGLUTININ TITRE
NHIgG
ALG
3x10⁸ s.r.b.c.
3x10⁸ s.r.b.c.
WEEKS AFTER PRIMARY CHALLENGE
0
2
4
6
8
10
5
10
15

ALG pretreatment the primary immune response to SRBC remained depressed for at least three months. However, this schedule of ALG treatment did not inhibit the sensitization of these animals, because a subsequent challenge with the antigen resulted in an immune response comparable with that in the control animals. These findings were confirmed by a similar experiment in C57Bl mice (Figure 10).

## IMMUNE RESPONSE TO PNEUMOCOCCAL POLYSACCHARIDE (SSS-III)

### Effect of standard ALG(1d) on the immune response of different strains of mice to SSS-III

Mice (4-6 per group) were injected intraperitoneally on days -4 and -2 with 0.25 ml. of 2 g. per cent (5 mg.) NHIgG or ALG. Two days later (day 0) they were challenged intravenously with 1µg. of purified SSS-III antigen. Five days after antigenic challenge the animals were exsanguinated and the spleens removed for PFC assays. The results of these assays are recorded in Table 24.

The magnitude of the immune response in different mouse strains varied considerably; the A/HeJ and C57Bl mice responded very poorly to this dose of the antigen whereas Balb/c, CBA and C3H mice gave relatively higher responses. Since all the mouse strains were not tested

FIGURE 10

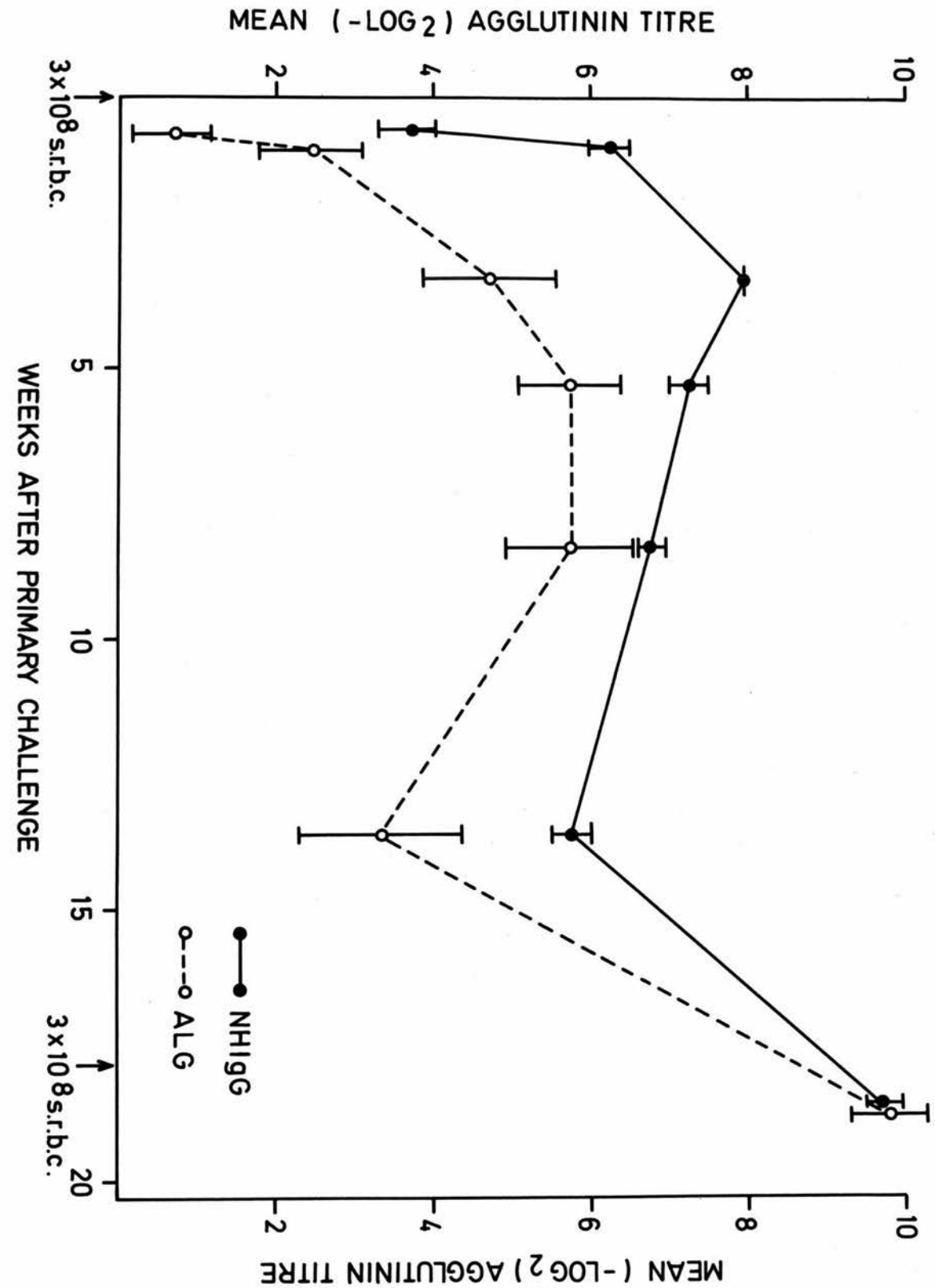

RECOVERY OF IMMUNOCOMPETENCE IN C57Bl MICE TREATED WITH ALG ON DAY -4 AND -2
MEAN (-LOG2) AGGLUTININ TITRE
2
4
6
8
10
3x10⁸ s.r.b.c.
5
10
15
20
3x10⁸ s.r.b.c.
WEEKS AFTER PRIMARY CHALLENGE
NHIgG
ALG

TABLE 24 - EFFECT OF ALG ON THE PRIMARY IMMUNE RESPONSE OF DIFFERENT MOUSE STRAINS AGAINST PNEUMOCOCCAL POLYSACCHARIDE (SSS-III)[a]

| Mouse Strain | Plaque Forming Cells Per Spleen[b] | | p[c] |
|---|---|---|---|
| | NHIgG | ALG | |
| A/HeJ | 11,209(8,040-15,625) (4)[d] | 222(186-265) (4) | <<0.001 |
| $C_{57}Bl$ | 1,058(805-1,391) (5) | 38(13-116) (5) | <0.02 |
| Balb/c[e] | 29,765(23,338-37,962) (6) | 975(602-1,580) (6) | <0.001 |
| | 15,190(11,789-19,572) (4) | 790(587-1,062) (5) | <0.001 |
| DBA/1 | 9,773(6,613-14,443) (4) | 561(473-665) (4) | <0.001 |
| CBA[e] | 12,254(9,393-15,986) (4) | 1,063(741-1,526) (4) | <0.005 |
| | 19,299(17,333-21,487) (4) | 576(452-734) (4) | <<0.001 |
| $C_3H$ | 21,035(17,752-24,924) (4) | 2,573(2,086-3,173) (6) | <0.001 |

(a) 5.0 mg. NHIgG or ALG injected i.p. on days -4 and -2; 1.0μg. SSS-III given i.v. on day 0 and tested on day 5.

(b) Geometric mean with the limits of one standard error.

(c) Comparison between NHIgG- and ALG-treated groups.

(d) Number of mice in each group.

(e) Observations from two different experiments.

at the same time, it is not possible to make an interstrain comparison from these results. Nevertheless, the interstrain variations were confirmed in another series of experiments and the relatively poor responsiveness of A/HeJ and C57Bl mice compared with that of Balb/c mice was established using a suboptimal dose (0.1μg.) of SSS-III antigen (see Appendix II). However, in all strains of mice, the immune response to this dose (1.0μg.) of SSS-III was significantly suppressed (P values of <0.02 - <0.001) by ALG pretreatment.

The serum agglutinin titres in these mice were also significantly suppressed by the ALG treatment in most mouse strains, although the reduction in the agglutinin titres was not as marked in some strains as in others (note A/HeJ, Balb/c and DBA/1 in Table 25). The suppression in A/HeJ mice was just below the significance level (P value just over 0.05) and the suppression in C57Bl mice was not at all significant. The latter may be due to very poor agglutinin response of this mouse strain. The suppression of the agglutinin responses in CBA and C3H mice was very marked and highly significant (P values less than 0.001).

## Effect of varying antigen dosage on the suppression of the response by ALG

Groups of mice (3-6 mice per group) were treated with NHIgG or ALG and challenged with 0.1 or 5μg. of SSS-III

TABLE 25 - THE EFFECT OF ALG ON THE PRIMARY HAEMAGGLUTININ RESPONSE OF DIFFERENT MOUSE STRAINS AGAINST PNEUMOCOCCUS POLYSACCHARIDE (SSS-III)[a]

| Mouse Strain | NHIgG | | ALG | | P[c] |
|---|---|---|---|---|---|
| | No. of mice | $Log_2$ Titre[b] | No. of mice | $Log_2$ Titre | |
| A/HeJ | 5 | 5.20 ± 0.20 | 5 | 4.20 ± 0.22 | N.S. |
| $C_{57}Bl$ | 5 | 2.00 ± 0.71 | 5 | 0.80 ± 0.37 | N.S. |
| Balb/c[d] | 6 | 7.67 ± 0.21 | 6 | 5.67 ± 0.21 | <0.001 |
| | 4 | 8.50 ± 0.29 | 5 | 4.60 ± 0.24 | <0.001 |
| DBA/1 | 4 | 5.25 ± 0.25 | 4 | 4.00 ± 00 | <0.005 |
| CBA[d] | 5 | 4.40 ± 0.24 | 5 | 0.20 ± 0.20 | <<0.001 |
| | 4 | 6.25 ± 0.25 | 4 | neg | <<0.001 |
| | 4 | 6.25 ± 0.25 | 4 | 2.5 ± 0.5 | <0.001 |
| $C_3H$ | 4 | 6.50 ± 0.29 | 6 | 1.50 ± 0.34 | <0.001 |

(a) 5.0 mg. NHIgG or ALG injected i.p. on days -4 and -2; 1.0μg. SSS-III given i.v. on day 0 and tested on day 5.

(b) Arithmetic mean ± 1 standard error.

(c) Comparison between NHIgG- and ALG-treated groups: P values greater than 0.05 were considered not significant (N.S.).

(d) Observations from different experiments.

(the schedule and the dose of ALG were described in the preceding experiments). Five days later the animals were sacrificed for splenic PFC and serum agglutinin assays. The results of these experiments have been documented in Tables 26 and 27.

It is apparent that lower doses of the antigen (0.1μg.) failed to give a significant response in some strains ($C_{57}Bl$ and DBA/1 in Table 26) or elicited only very small response (e.g. A/HeJ and DBA/1 mice). However, in the strains in which there was a satisfactory response to this dose of antigen, it was significantly suppressed by ALG pretreatment (all strains except $C_{57}Bl$ and DBA/1 in Table 26). In addition, the response to 5μg. SSS-III was invariably suppressed by ALG in all strains and, in all cases, the suppression was statistically significant. It should, however, be noted that in all cases the degree of suppression in mice given 5μg. SSS-III was less marked than that in mice given 1.0μg. SSS-III (compare Tables 24 and 26). The serum agglutinin responses to both antigen doses were suppressed by the ALG treatment in all mouse strains, with the exception of $C_{57}Bl$ and DBA/1 mice. In the latter two strains the ALG failed to suppress significantly the agglutinin response to the lower antigen dose. ALG also failed to suppress the agglutinin response to 5μg. SSS-III in A/HeJ mice andin one experiment it failed to suppress the response of Balb/c mice to 0.1μg. SSS-III; the P value

TABLE 26 - THE EFFECT OF ANTIGEN DOSE ON THE SUPPRESSION OF THE PRIMARY IMMUNE RESPONSE BY ALG IN DIFFERENT MOUSE STRAINS AGAINST PNEUMOCOCCAL POLYSACCHARIDE (SSS-III)[a]

| Mouse Strain | Ag Dose µg. | NHIgG | | ALG | | P[c] |
|---|---|---|---|---|---|---|
| | | No. of Mice | PFC/Spleen[b] | No. of Mice | PFC/Spleen[b] | |
| A/HeJ | 0.1 | 5 | 1,271 (747-2,163) | 6 | 68 (44-107) | <0.005 |
| | 5.0 | 6 | 7,270 (5,563-9,500) | 6 | 2,087 (1,470-2,962) | <0.02 |
| $C_{57}Bl$ | 0.1 | 5 | 145 (105-197) | 5 | 122 (86-172) | N.S. |
| | 5.0 | 5 | 9,798 (7,980-12,030) | 5 | 1,325 (868-2,023) | <0.005 |
| Balb/c | 0.1[d] | 3 | 5,746 (4,931-6,696) | 3 | 1,925 (1,506-2,460) | <0.02 |
| | | 3 | 2,565 (2,152-3,056) | 3 | 282 (111-716) | N.S. |
| | 5.0 | 5 | 17,679 (13,729-22,766) | 5 | 1,340 (866-2,074) | <0.001 |
| DBA/1 | 0.1[d] | 4 | 1,108 (865-1,420) | 4 | 869 (670-1,126) | N.S. |
| | | 4 | 220 (173-280) | 4 | 243 (216-274) | N.S. |
| | 5.0 | 5 | 11,101 (8,426-14,626) | 4 | 2,819 (1,682-4,725) | <0.05 |
| CBA | 0.1 | 5 | 2,914 (2,350-3,613) | 6 | 1,123 (853-1,478) | <0.025 |
| | 5.0 | 5 | 22,228 (19,137-25,818) | 5 | 859 (730-1,010) | <<0.001 |
| $C_3H$ | 0.1 | 4 | 2,068 (1,543-2,771) | 4 | 471 (338-656) | <0.02 |
| | 5.0 | 5 | 37,204 (29,425-47,040) | 7 | 4,217 (2,865-6,210) | <0.005 |

(a) 5.0 mg. NHIgG or ALG injected i.p. on days -4 and -2; 0.1 or 5.0µg. SSS-III given i.v. on day 0 and tested on day 5.

(b) Geometric mean with the limits of one standard error.

(c) Comparison between NHIgG- and ALG-treated groups: P values greater than 0.05 were considered not significant (N.S.)

(d) Observations from two different experiments.

TABLE 27 - THE EFFECT OF ANTIGEN DOSE ON THE SUPPRESSION OF THE PRIMARY HAEMAGGLUTININ RESPONSE BY ALG IN DIFFERENT MOUSE STRAINS AGAINST PNEUMOCOCCAL POLYSACCHARIDE (SSS-III)[a]

| Mouse Strain | Ag Dose µg. | NHIgG | | ALG | | P[c] |
|---|---|---|---|---|---|---|
| | | No. of mice | $Log_2$ Titre[b] | No. of mice | $Log_2$ Titre | |
| A/HeJ | 0.1[d] | 5 | 6.20 ± 0.2 | 5 | 3.50 ± 0.72 | <0.01 |
| | | 6 | 5.33 ± 0.42 | 6 | 3.83 ± 0.17 | <0.01 |
| | 5.0 | 6 | 4.67 ± 0.33 | 6 | 4.67 ± 0.33 | N.S. |
| $C_{57}Bl$ | 0.1[d] | 5 | 3.20 ± 0.58 | 5 | 1.60 ± 0.51 | N.S. |
| | | 6 | 4.33 ± 0.33 | 6 | 3.67 ± 0.71 | N.S. |
| | 5.0 | 5 | 3.80 ± 1.02 | 5 | 0.40 ± 0.40 | <0.02 |
| Balb/c | 0.1[d] | 3 | 7.67 ± 0.33 | 3 | 4.33 ± 0.33 | <0.005 |
| | | 3 | 7.67 ± 0.67 | 3 | 5.67 ± 0.67 | N.S. |
| | 5.0 | 6 | 6.80 ± 0.37 | 5 | 3.60 ± 0.93 | <0.05 |
| DBA/1 | 0.1[d] | 4 | 5.25 ± 0.25 | 4 | 5.00 ± 0.41 | N.S. |
| | | 4 | 5.75 ± 0.25 | 4 | 5.25 ± 0.25 | N.S. |
| | 5.0 | 5 | 5.80 ± 0.20 | 4 | 4.5 ± 0.29 | <0.01 |
| CBA | 0.1[d] | 8 | 3.37 ± 0.26 | 15 | 1.80 ± 0.28 | <0.005 |
| | | 5 | 5.00 ± 0.49 | 5 | 2.60 ± 0.51 | <0.02 |
| | | 5 | 5.40 ± 0.24 | 6 | 0.33 ± 0.21 | <<0.001 |
| | 5.0 | 5 | 5.00 ± 1.00 | 5 | neg | <0.005 |
| $C_3H$ | 0.1 | 4 | 6.25 ± 0.25 | 4 | 2.75 ± 1.03 | <0.02 |
| | 5.0 | 5 | 6.40 ± 0.24 | 7 | 1.57 ± 0.37 | <0.001 |

(a) 5.0 mg. NHIgG or ALG injected i.p. on days -4 and -2; 0.1 or 5.0µg. SSS-III given i.v. on day 0 and tested on day 5.

(b) Arithmetic mean ± 1 standard error.

(c) Comparison between NHIgG- and ALG-treated groups: P values greater than 0.05 were considered not significant (N.S.).

(d) Observations from different experiments.

in the latter case was only marginally higher than the significance level (Table 27).

Effect of different ALG preparations on the immune response to SSS-III

Groups of mice containing 4-5 animals per group were injected intraperitoneally with 5 mg. of NHIgG or individual ALG preparations on days -4 and -2 and were challenged on day 0 with 1.0μg. SSS-III antigen. The spleens from these mice were tested for the PFC response on the 5th day after the antigenic challenge and the sera were tested for agglutinating antibodies.

The results of the splenic PFC responses have been summarized in Table 28. It will be observed that only ALG preparation 1d produced a suppression of anti-SSS-III response in CBA mice which was statistically significant. Although suppression was caused by preparations 2 and 3, the p values in these instances were marginally outside the significance limits. Indeed one preparation (ALG 4) produced a significant increase in the number of PFC per spleen. However, when the results were expressed as the number of PFC per $10^6$ nucleated spleen cells (see Table 29) it appears that ALG preparations 2 and 3 significantly suppressed the response. Furthermore, the enhancement caused by the ALG preparation 4 proved to be statistically non-significant when the PFC per $10^6$ nucleated spleen cells were considered.

TABLE 28 - THE EFFECT OF VARIOUS ALG PREPARATIONS ON THE IMMUNE RESPONSE OF CBA AND Balb/c MICE AGAINST PNEUMOCOCCAL POLYSACCHARIDE (SSS-III)[a]

| Mouse Strain | NHIgG | | ALG | | | P[c] |
|---|---|---|---|---|---|---|
| | No. of Mice | PFC/Spleen[b] | Prep. | No. of Mice | PFC/Spleen | |
| Balb/c | 4 | 15,190 (11,789-19,572 | 1d | 5 | 790 (587-1,062) | <0.001 |
| | 4 | 24,459 (18,495-32,345) | 2 | 4 | 5,377 (3,705-7,802) | <0.02 |
| | 4 | 24,459 (18,495-32,345) | 3 | 5 | 2,416 (1,969-2,965) | <0.001 |
| | 4 | 15,190 (11,789-19,572) | 4 | 5 | 6,673 (5,321-8,369) | <0.05 |
| CBA | 4 | 19,299 (17,333-21,487) | 1d | 4 | 576 (452-734) | <<0.001 |
| | 4 | 6,930 (4,723-10,167) | 2 | 4 | 2,803 (2,419-3,249) | N.S. |
| | 5 | 12,154 (10,369-14,247) | 3 | 5 | 8,359 (7,612-9,180) | N.S. |
| | 5 | 12,154 (10,369-14,247) | 4 | 4 | 26,482 (20,913-33,535) | <0.025 |

(a) 5.0 mg. NHIgG or ALG injected i.p. on days -4 and -2; 1.0μg. SSS-III given i.v. on day 0 and tested on day 5.

(b) Geometric mean with the limits of one standard error.

(c) Comparison between NHIgG- and ALG-treated groups: P values greater than 0.05 were considered not significant (N.S.).

TABLE 29 - THE EFFECT OF VARIOUS ALG PREPARATIONS ON THE IMMUNE RESPONSE OF CBA AND Balb/c MICE AGAINST PNEUMOCOCCAL POLYSACCHARIDE (SSS-III)[a]

| Mouse Strain | NHIgG | | ALG | | | P[c] |
|---|---|---|---|---|---|---|
| | No. of Mice | PFC per $10^6$ Spleen Cells[b] | Prep. No. | No. of Mice | PFC per $10^6$ Spleen Cells | |
| Balb/c | 4 | 50.30 (39.76-63.65) | 1d | 5 | 0.77 (0.61-0.99) | $<<0.001$ |
| | 4 | 108.88 (89.89-131.88) | 2 | 4 | 15.62 (10.82-22.56) | $<0.005$ |
| | 4 | 108.88 (89.89-131.88) | 3 | 5 | 4.84 (4.00-5.86) | $<0.001$ |
| | 4 | 50.30 (39.76-63.65) | 4 | 5 | 8.48 (6.54-10.99) | $<0.005$ |
| CBA | 4 | 110.39 (103.33-117.92) | 1d | 4 | 0.69 (0.51-0.95) | $<<0.001$ |
| | 4 | 40.04 (21.01-61.64) | 2 | 4 | 15.81 (12.55-19.93) | N.S. |
| | 5 | 75.20 (66.13-85.52) | 3 | 5 | 39.50 (38.04-41.01) | $<0.005$ |
| | 5 | 75.20 (66.13-85.52) | 4 | 4 | 95.79 (71.70-127.96) | N.S. |

(a) 5.0 mg. NHIgG or ALG injected i.p. on days -4 and -2; 1.0 µg. SSS-III given i.v. on day 0 and tested on day 5.

(b) Geometric mean with the limits of one standard error.

(c) Comparison between NHIgG- and ALG-treated groups: P values greater than 0.05 were considered not significant (N.S.).

In contrast all four preparations significantly suppressed the plaque forming cell response to SSS-III in Balb/c mice. This was statistically significant both at the PFC per spleen (Table 28) and the PFC per $10^6$ nucleated spleen cell levels (Table 29).

The circulating antibody levels were also suppressed by the ALG treatment but the effect was probably less dramatic than observed in the PFC assays (Table 30). Nevertheless a significant suppression of agglutinin responses in Balb/c mice was obtained with all ALG preparations while only preparation 4 failed to significantly suppress the agglutinin response in CBA mice.

## Effect of ALG prepared from successive bleeds from a single horse

In these experiments groups of animals (4-5 mice per group) were pretreated with four different ALG preparations isolated from successive bleeds obtained from a single horse at various stages of immunization (see Table 2). The control animals received 5 mg. NHIgG whereas the test ones were given the same quantity of ALG on days -4 and -2. The mice in all groups received 1.0µg. SSS-III on day 0.

From the results of PFC assays carried out on day 5 after challenge it is apparent that with the exception of ALG preparation 1a (obtained after a single injection of the horse) all preparations significantly suppressed the

TABLE 30 - THE EFFECT OF VARIOUS ALG PREPARATIONS ON THE HAEMAGGLUTININ RESPONSE OF CBA AND Balb/c MICE AGAINST PNEUMOCOCCAL POLYSACCHARIDE (SSS-III)[a]

| Mouse Strain | NHIgG | | ALG | | | P[c] |
|---|---|---|---|---|---|---|
| | No. of mice | $Log_2$ Titre[b] | Prep. No. | No. of mice | $Log_2$ Titre | |
| Balb/c | 4 | 8.50 ± 0.29 | 1d | 5 | 4.60 ± 0.24 | <0.001 |
| | 4 | 7.50 ± 0.29 | 2 | 4 | 6.25 ± 0.25 | <0.02 |
| | 4 | 7.50 ± 0.29 | 3 | 4 | 5.75 ± 0.48 | <0.02 |
| | 4 | 8.50 ± 0.29 | 4 | 5 | 6.40 ± 0.4 | <0.005 |
| CBA | 4 | 6.25 ± 0.25 | 1d | 4 | 2.50 ± 0.50 | <0.001 |
| | 4 | 6.25 ± 0.25 | 2 | 4 | 3.75 ± 0.25 | <0.001 |
| | 5 | 6.80 ± 0.37 | 3 | 5 | 5.20 ± 0.20 | <0.01 |
| | 5 | 6.60 ± 0.24 | 4 | 4 | 5.50 ± 0.64 | N.S. |

(a) 5.0 mg. NHIgG or ALG injected i.p. on days -4 and -2; 1.0 μg. SSS-III given i.v. on day 0 and tested on day 5.

(b) Arithmetic mean ± 1 standard error.

(c) Comparison between NHIgG- and ALG-treated groups: P values greater than 0.05 were considered not significant (N.S.).

TABLE 31 - EFFECT OF HYPERIMMUNIZATION OF ALS DONOR ON THE ABILITY OF THE ALG TO SUPPRESS THE IMMUNE RESPONSE OF Balb/c AND CBA MICE AGAINST PNEUMOCOCCAL POLYSACCHARIDE (SSS-III)[a]

| Mouse Strain | NHIgG | | ALG | | | P[c] |
|---|---|---|---|---|---|---|
| | No. of Mice | PFC/Spleen[b] | Prep. No. | No. of Mice | PFC/Spleen | |
| Balb/c | 4 | 10,439 (8,519-12,792) | 1b | 5 | 3,240 (2,429-4,323) | <0.02 |
| | 4 | 10,439 (8,519-12,792) | 1c | 5 | 2,884 (2,036-4,085) | <0.025 |
| | 4 | 15,190 (11,789-19,572) | 1d | 5 | 790 (587-1,062) | <0.001 |
| CBA | 5 | 12,154 (10,369-14,247) | 1a | 5 | 17,878 (15,260-20,946) | N.S. |
| | 4 | 19,299 (17,333-21,487) | 1b | 5 | 4,614 (3,239-6,572) | <0.02 |
| | 4 | 19,299 (17,333-21,487) | 1c | 5 | 1,795 (1,374-2,346) | <0.001 |
| | 4 | 19,299 (17,333-21,487) | 1d | 4 | 576 (452-734) | <<0.001 |

(a) 5.0 mg. NHIgG or ALG injected i.p. on days -4 and -2; 1.0μg. SSS-III given i.v. on day 0 and tested on day 5.

(b) Geometric mean with the limits of one standard error.

(c) Comparison between NHIgG- and ALG-treated groups: P values greater than 0.05 were considered not significant (N.S.).

PFC response of CBA mice to 1.0µg. SSS-III (Preps. 1b, 1c and 1d in Table 31). Similarly, preparations 1b-1d caused a significant suppression of this response in Balb/c mice. In contrast preparation 1a caused a slight enhancement of the anti-SSS-III response though this was not statistically significant. It should be noted that there was a gradual increase in the immunosuppressive capacity of ALG with hyperimmunization of the horse.

The data from the same experiment have been recorded as PFC per $10^6$ nucleated spleen cells in Table 32. Expressed in this form the suppression of anti-SSS-III response by various ALG preparations in both CBA and Balb/c mice is more obvious. However, ALG 1a still fails to show any immunosuppressive effect.

The results of serological tests on these mice were somewhat different. The ALG preparation 1b failed to suppress significantly the agglutinin response in both CBA and Balb/c mice, whereas all other preparations suppressed significantly the anti-SSS-III agglutinin response in both Balb/c and CBA mice (Table 33).

## Effect of an alternative ALG treatment schedule on its immunosuppressive capacity

The results of studies on the effect of ALG on SSS-III responses presented in the previous sections contrast markedly with the observations of Baker and his colleagues (Baker,

TABLE 32 - THE EFFECT OF HYPERIMMUNIZATION OF ALS DONOR ON THE ABILITY OF THE ALG TO SUPPRESS THE IMMUNE RESPONSE OF Balb/c AND CBA MICE AGAINST PNEUMOCOCCAL POLYSACCHARIDE (SSS-III)[a]

| Mouse Strain | NHIgG | | ALG | | | P[c] |
|---|---|---|---|---|---|---|
| | No. of Mice | PFC/$10^6$ Spleen Cells[b] | Prep. No. | No. of Mice | PFC/$10^6$ Spleen Cells | |
| Balb/c | 4 | 45.99 (37.76-56.00) | 1b | 5 | 11.84 (9.05-15.49) | <0.001 |
| | 4 | 45.99 (37.76-56.00) | 1c | 5 | 11.28 (7.84-16.24) | <0.001 |
| | 4 | 50.30 (39.76-63.65) | 1d | 5 | 0.77 (0.61-0.99) | <<0.001 |
| CBA | 4 | 75.20 (66.13-85.52) | 1a | 5 | 89.71 (80.81-99.59) | N.S. |
| | 4 | 110.39 (103.33-117.92) | 1b | 5 | 16.54 (10.95-24.64) | <0.005 |
| | 4 | 110.39 (103.33-117.92) | 1c | 5 | 7.03 (5.18-9.51) | <0.001 |
| | 4 | 110.39 (103.33-117.92) | 1d | 4 | 0.69 (0.51-0.95) | <<0.001 |

(a) 5.0 mg. NHIgG or ALG injected i.p. on days -4 and -2; 1.0μg. SSS-III given i.v. on day 0 and tested on day 5.

(b) Geometric mean with the limits of one standard error.

(c) Comparison between NHIgG- and ALG-treated groups: P values greater than 0.05 were considered not significant (N.S.).

TABLE 33 - THE EFFECT OF HYPERIMMUNIZATION OF ALS DONOR ON THE ABILITY OF THE ALG TO SUPPRESS THE HAEMAGGLUTININ RESPONSE OF Balb/c AND CBA MICE AGAINST PNEUMOCOCCAL POLYSACCHARIDE (SSS-III)a

| Mouse Strain | NHIgG | | ALG | | | P[c] |
|---|---|---|---|---|---|---|
| | No. of Mice | $Log_2$ Titre[b] | Prep. No. | No. of Mice | $Log_2$ Titre | |
| Balb/c | 4 | 7.50 ± 0.29 | 1b | 5 | 6.80 ± 0.37 | N.S. |
| | 4 | 7.50 ± 0.29 | 1c | 5 | 5.60 ± 0.24 | <0.005 |
| | 4 | 8.50 ± 0.29 | 1d | 5 | 4.60 ± 0.24 | <0.001 |
| CBA | 5 | 6.80 ± 0.37 | 1a* | 5 | 5.20 ± 0.2 | <0.01 |
| | 5 | 6.60 ± 0.24 | | 5 | 5.80 ± 0.20 | <0.05 |
| | 4 | 6.25 ± 0.25 | 1b | 5 | 3.60 ± 1.01 | N.S. |
| | 4 | 6.25 ± 0.25 | 1c | 5 | 3.40 ± 0.51 | <0.005 |
| | 4 | 6.25 ± 0.25 | 1d* | 4 | 2.50 ± 0.50 | <0.001 |
| | 4 | 6.25 ± 0.25 | | 4 | neg | <<0.001 |

(a) 5.0 mg. NHIgG or ALG injected i.p. on days -4 and -2; 1.0 µg. SSS-III given i.v. on day 0 and tested on day 5.

(b) Arithmetic mean ± 1 standard error.

(c) Comparison between NHIgG- and ALG-treated groups: P values greater than 0.05 were considered not significant (N.S.).

* Observation from two different experiments.

Barth, Stashak and Amsbaugh, 1970; Baker, Stashak, Amsbaugh, Prescott and Barth, 1970) who demonstrated that their anti-lymphocyte antibody potentiated rather than suppressed the immune response to SSS-III antigen. It was conceivable that these contrasting observations could be attributed to differences in the ALG treatment schedules used. In order to test this possibility groups of Balb/c mice were treated with 5 mg. of NHIgG or ALG preparations and challenged with 1.0µg. SSS-III following the schedule described in the preceding sections. Other groups were treated with 5 mg. of the same NHIgG or ALG preparations 15-20 minutes prior to antigenic challenge with an identical dose of SSS-III (a schedule similar to that adopted by Baker and his colleagues). The animals were sacrificed five days after the antigenic challenge and splenic PFC and circulating agglutinin responses measured. In addition groups of CBA mice were treated with NHIgG or ALG on day -8 and -6 or -4 and -2, challenged with SSS-III on day 0 and their response measured on day 5. The results obtained are recorded in Tables 34-36.

As previously noted (see Balb/c in Tables 31 and 32) all ALG preparations tested suppressed the PFC response of Balb/c mice to SSS-III if administered on days -4 and -2 prior to antigenic challenge. In contrast however, if their administration was delayed until just prior to antigen administration only ALG 1d was effective: Indeed

TABLE 34 - THE EFFECT OF ALTERNATIVE ANTI-LYMPHOCYTIC ANTIBODY TREATMENT SCHEDULES ON THE IMMUNE RESPONSE OF MICE AGAINST PNEUMOCOCCAL POLYSACCHARIDE (SSS-III)[a]

| ALG Prep. | Schedule[a] | Plaque Forming Cells Per Spleen[b] | | P[c] |
|---|---|---|---|---|
| | | NHIgG | ALG | |
| 1d | -4; -2 | 15,190 (11,789-19,572) (4)[d] | 790 (587-1,062) (5) | <0.001 |
| | 0 | 24,456 (21,635-27,645) (4) | 3,067 (2,520-3,733) (5) | <0.001 |
| 1d* | -4; -2 | 9,590 (8,910-10,323) (4) | 170 (115-253) (4) | <0.001 |
| | -8; -6 | 5,110 (4,807-5,431) (3) | 145 (78-269) (4) | <0.01 |
| 2 | -4; -2 | 24,459 (18,495-32,345) (4) | 5,377 (3,705-7,802) (4) | <0.02 |
| | 0 | 22,259 (19,716-25,130) (4) | 27,434 (19,491-38,613) (4) | N.S. |
| 3 | -4; -2 | 24,459 (18,495-32,345) (4) | 2,416 (1,969-2,965) (5) | <0.001 |
| | 0 | 22,259 (19,716-25,130) (4) | 18,075 (12,635-25,858) (5) | N.S. |
| 4 | -4; -2 | 15,190 (11,789-19,572) (4) | 6,673 (5,321-8,369) (5) | <0.05 |
| | 0 | 24,456 (21,635-27,645) (4) | 19,585 (15,027-25,527) (5) | N.S. |

(a) 5 mg. ALG injected i.p. on days -8, -6, -4, -2 or on day 0; 1.0µg. SSS-III given i.v. on day 0 and tested on day 5.

(b) Geometric mean with the limits of one standard error.

(c) Comparison between NHIgG- and ALG-treated groups: P values greater than 0.05 were considered not significant (N.S.).

(d) Number of mice in each group.

* Tested in CBA mice; all other observations were made in Balb/c mice.

ALG 2 potentiated the response although this potentiation was not statistically significant (Table 34). The data on PFC per $10^6$ nucleated spleen cells recorded in Table 35 indicate a slightly better suppression by thevarious ALG preparations. ALG preparations 1d and 3 significantly suppressed the SSS-III response even when administered just before the antigen; preparations 2 and 4 still remained ineffective.

Incidentally, it should also be noted (see following section) that ALG 1d was effective at suppressing PFC response of CBA mice when administered on days -8 and -6 before the antigen, although the effect was not as marked as that when the ALG was administered on days -4 and -2 (Table 35).

The results of serological tests recorded in Table 36 were again slightly inconsistent with those of PFC assays. In this test ALG preparations 1d and 2 failed to suppress the agglutinin responses, whereas the remaining preparations were significantly effective at suppressing agglutinin titres. It should be noted that the suppression by ALG 1d administered on days -8 and -6 before the antigen was just outside the significance limits (Table 36).

## Immunocompetence of ALG treated mice reconstituted with various syngeneic lymphoid cell populations

These preliminary experiments were performed to establish

TABLE 35 - THE EFFECT OF ALTERNATIVE ANTI-LYMPHOCYTIC ANTIBODY TREATMENT SCHEDULES ON THE IMMUNE RESPONSE OF MICE AGAINST PNEUMOCOCCAL POLYSACCHARIDE (SSS-III)[a]

| ALG Prep. | Schedule[a] | NHIgG | | ALG | | P[c] |
|---|---|---|---|---|---|---|
| | | No. of Mice | PFC/$10^6$ Spleen Cells[b] | No. of Mice | PFC/$10^6$ Spleen Cells[b] | |
| 1d | -4; -2 | 4 | 50.30 (39.76-63.65) | 5 | 0.77 (0.61-0.99) | $<<0.001$ |
| | 0 | 4 | 91.95 (78.07-108.29) | 5 | 4.71 (3.70-5.98) | $<0.005$ |
| 1d* | -4; -2 | 4 | 55.73 (50.44-61.57) | 4 | 0.58 (0.35-0.95) | $<0.001$ |
| | -8; -6 | 3 | 31.00 (29.04-33.10) | 5 | 0.80 (0.43-1.49) | $<0.005$ |
| 2 | -4; -2 | 4 | 108.88 (89.89-131.88) | 4 | 15.62 (10.82-22.56) | $<0.005$ |
| | 0 | 4 | 83.69 (70.88-98.82) | 4 | 108.92 (78.20-151.70) | N.S. |
| 3 | -4; -2 | 4 | 108.88 (89.89-131.88) | 5 | 4.84 (4.00-5.86) | $<<0.001$ |
| | 0 | 4 | 83.69 (70.88-98.82) | 5 | 62.65 (41.19-95.29) | N.S. |
| 4 | -4; -2 | 4 | 50.30 (39.76-63.65) | 5 | 8.48 (6.54-10.99) | $<0.005$ |
| | 0 | 4 | 91.95 (78.07-108.29) | 5 | 52.34 (31.20-66.49) | N.S. |

(a) 5 mg. ALG injected i.p. on days -8, -6, -4, -2 or on day 0; 1.0µg. SSS-III given i.v. on day 0 and tested on day 5.

(b) Geometric mean with the limits of one standard error.

(c) Comparison between NHIgG- and ALG-treated groups: P values greater than 0.05 were considered not significant (N.S.).

* Tested in CBA mice; all other observations were made in Balb/c mice.

TABLE 36 - THE EFFECT OF ALTERNATIVE ALG TREATMENT SCHEDULES ON THE HAEMAGGLUTININ RESPONSE OF MICE AGAINST PNEUMOCOCCAL POLYSACCHARIDE (SSS-III)[a]

| ALG Prep. | Schedule[a] | NHIgG | | ALG | | P[c] |
|---|---|---|---|---|---|---|
| | | No. of Mice | $Log_2$ Titre[b] | No. of Mice | $Log_2$ Titre | |
| 1d | -4; -2 | 4 | 8.50 ± 0.29 | 5 | 4.60 ± 0.24 | <0.001 |
| | 0 | 4 | 8.25 ± 0.48 | 5 | 8.80 ± 0.20 | N.S. |
| 1d* | -4; -2 | 3 | 6.25 ± 0.25 | 4 | neg | <<0.001 |
| | -8; -6 | 3 | 6.33 ± 0.33 | 5 | 5.40 ± 0.24 | N.S. |
| 2 | -4; -2 | 4 | 7.50 ± 0.29 | 4 | 6.25 ± 0.25 | <0.02 |
| | 0 | 4 | 8.75 ± 0.48 | 4 | 8.75 ± 0.25 | N.S. |
| 3 | -4; -2 | 4 | 7.50 ± 0.29 | 4 | 5.75 ± 0.48 | <0.025 |
| | 0 | 4 | 8.75 ± 0.48 | 4 | 7.25 ± 0.25 | <0.05 |
| 4 | -4; -2 | 4 | 8.50 ± 0.29 | 5 | 6.40 ± 0.40 | <0.005 |
| | 0 | 4 | 8.25 ± 0.48 | 5 | 9.80 ± 0.80 | N.S. |

(a) 5 mg. ALG injected i.p. on days -8, -6, -4, -2 or on day 0; 1.0µg. SSS-III given i.v. on day 0 and tested on day 5.

(b) Arithmetic mean ± 1 standard error.

(c) Comparison between NHIgG- and ALG-treated groups: P values greater than 0.05 were considered not significant (N.S.).

* Tested in CBA mice; all other observations were made in Balb/c mice.

the cellular basis of the immunological deficiency in the mice treated with ALG preparation ld. In these studies CBA mice were injected intraperitoneally on days -8 and -6 with 0.25 ml. of 2 g. per cent (5 mg.) NHIgG (group A) or ALG (groups B-E) preparation. Four days later (i.e. on day -2) the mice were injected intravenously with either Eagle's medium alone (groups A and B), syngeneic thymocytes (group C), syngeneic bone marrow cells (group D) or syngeneic spleen cells (group E). The number of lymphoid cells injected in each group is indicated in Table 37. Two days after cellular reconstitution (day 0), all the mice were challenged with 1.0μg. SSS-III antigen. The animals were sacrificed five days later and the splenic PFC responses were measured.

It is apparent from the data presented in Table 37 that mice receiving Eagle's medium alone (group B) after ALG treatment were only able to mount a meagre response to SSS-III. However, reconstitution with normal thymocytes (group C), bone marrow cells (group D) or spleen cells (group E) resulted in a partial recovery of the capacity to respond to SSS-III.

TABLE 37 - THE ANTI-SSS-III RESPONSE OF ANTI-LYMPHOCYTIC TREATED CBA MICE RECONSTITUTED WITH VARIOUS SYNGENEIC LYMPHOID CELL POPULATIONS[a]

| Group | Treatment | Reconstitution with Eagles medium containing | No. of Animals | PFC Per Spleen[b] | P | PFC/$10^6$ nucleated spleen cells[b] | P |
|---|---|---|---|---|---|---|---|
| A | Normal IgG | Nothing | 4 | 8,585 (7,522-9,798) | | 45.34 (41.33-49.74) | |
| B | ALG | Nothing | 5 | 642 (445-926) | A ≡ B <0.001 | 2.96 (2.13-7.15) | A ≡ B <0.001 |
| C | ALG | 32 x $10^6$ Thymocytes | 4 | 3,655 (2,826-4,728) | B ≡ C <0.01 | 15.35 (12.54-18.80) | B ≡ C <0.001 |
| D | ALG | 27 x $10^6$ Bone marrow cells | 5 | 2,594 (1,760-3,823) | B ≡ D <0.05 | 11.92 (8.31-17.11) | B ≡ D <0.001 |
| E | ALG | 28 x $10^6$ Spleen cells | 5 | 1,971 (1,448-2,681) | B ≡ E <0.05 | 8.52 (6.24-11.64) | B ≡ E <0.005 |

(a) 0.5 mg. NHIgG or ALG injected i.p. on days -8 and -6; lymphoid cells injected i.v. on day -2; 1.0 µg. SSS-III injected i.p. on day 0 and tested on day 5.

(b) Geometric mean with the limits of one standard error.

## IMMUNE RESPONSE TO BOVINE SERUM ALBUMIN (BSA)

### Effect of different ALG preparations on the immune response to BSA

Groups containing 5-6 CBA mice were injected intraperitoneally with 0.25 ml. of 2 g. per cent (5 mg.) NHIgG or ALG on days -4 and -2 and challenged intraperitoneally with 1 mg. alum-precipitated BSA. Mice were exsanguinated on day 21 after the challenge and the sera were collected. Sera from individual mice were tested for antibodies against BSA. The results of these tests have been summarized in Table 38.

It is clearly apparent that all ALG preparations tested were able to suppress the primary immune response to BSA. This included preparation 1a isolated from a bleed obtained after immunization with a single thymocyte inoculum. However, it can be seen that the degree of suppression varied from one preparation to the other. Also, from the comparison of the degree of suppression caused by ALG from different bleeds, it is apparent that the ability of these preparations to suppress anti-BSA response increased with hyperimmunization of the horse (compare ALG preparations 1a-1d in Table 38). It should, however, be noted that none of the ALG preparations significantly altered the relative binding affinity of antibody produced by these animals.

TABLE 38 - THE EFFECT OF DIFFERENT ALG PREPARATIONS ON THE IMMUNE RESPONSE OF CBA MICE AGAINST ALUM-BSA[a]

| ALG Prep. | No. of Mice | Antigen Binding Capacity[b] | P[c] | Relative Binding Affinity[d] | P[c] |
|---|---|---|---|---|---|
| NHIgG | 5 | 6.16(5.66-6.69) | | 37.0 ± 4.7 | |
| ALG1a | 6 | 1.99(1.76-2.25) | <0.001 | 35.4 ± 3.2 | N.S. |
| ALG1b | 6 | 1.66(1.39-1.99) | <0.001 | 28.3 ± 1.4 | N.S. |
| ALG1c | 6 | 0.81(0.61-1.07) | <0.001 | 35.5 ± 2.8 | N.S. |
| ALG1d | 6 | 0.80(0.54-1.19) | <0.005 | 30.9 ± 3.1 | N.S. |
| ALG2 | 5 | 2.50(2.25-2.78) | <0.001 | 39.0 ± 4.4 | N.S. |
| ALG3 | 6 | 1.62(1.48-1.77) | <<0.001 | 30.6 ± 2.3 | N.S. |
| ALG4 | 6 | 1.11(0.93-1.33) | <<0.001 | 33.3 ± 3.0 | N.S. |

(a) 5 mg. NHIgG or ALG injected i.p. on days -4 and -2; 1.0 mg. alum-BSA given i.p. on day 0 and tested on day 20.

(b) Geometric mean with the limits of one standard error.

(c) Comparison between NHIgG- and ALG-treated groups: P values greater than 0.05 were considered not significant (N.S.).

(d) Arithmetic mean ± 1 standard error.

# DISCUSSION

Observations made over the last decade have unequivocally demonstrated the effectiveness of ALG in suppressing allograft immunity. In addition various other cell mediated responses have been shown to be suppressed by ALG treatment (see the Introduction). It has been postulated that ALG caused the suppression of cell mediated immunity by eliminating or inactivating population of cells which are mainly implicated in the homograft and other cell mediated immune reactions (reviewed in Introduction).

The earlier findings of Lance and Batchelor (1968) indicated that the allograft response of mice could be suppressed by anti-lymphocytic serum while their antibody response to the same transplantation antigens was unaffected. On the basis of these experiments and some others in which the suppression of humoral immune response to Salmonella typhi 'H' antigen and in certain cases the immune response to BSA seemed refractive to the action of anti-lymphocytic serum, it was suggested that this reagent could selectively suppress cell mediated immunity by discriminately depleting the thymus-derived cells (Levey and Medawar, 1966a; Lance and Batchelor, 1968; Lance, 1968a,b). The role of thymus-derived lymphocytes in cell mediated immune reactions has already been stressed in the introductory part of this thesis. It should also be added that the recirculating pool of long-lived small lymphocytes which have been shown to be preferentially depleted by the ALG

treatment (Denman, Denman, Embling, 1968; Taub, 1969; Everett, Schwartz, Tyler and Perkins, 1970) have also been demonstrated to be chiefly derived from the thymus (Gowans and McGregor, 1965; Doenhoff, 1971).

In contrast, a number of observations have indicated that certain humoral immune responses could be suppressed by anti-lymphocytic antibody. This suppression could possibly be attributed to the elimination of thymus-derived lymphoid cell populations which have in the past decade been shown to play a vital role in the initiation of an immune response to a variety of antigens (Taylor, 1969; Roitt, Greaves, Torrigiani, Brostoff and Playfair, 1969; Playfair, 1971; Miller, Basten, Sprent and Cheers, 1971).

In the course of the early attempts to suppress the humoral immune responses by the anti-lymphocyte serum treatment, it was observed that adult thymectomy potentiated the immunosuppression caused by ALS (Jeejeebhoy, 1965a,b; Monaco, Wood and Russell, 1965; Leuchars, Wallis and Davies, 1968). These observations further strengthened the view that ALG could cause the suppression of different immune responses by eliminating, inactivating or interfering with the function of the thymus-derived cell population.

The site of action of ALG was investigated by Martin and Miller (1968). These authors concluded that ALS exerted its immunosuppressive effect in humoral immune systems by eliminating the thymus-derived antigen reactive

cells. These observations were confirmed and further extended by those of Mitchison (1970).

The observations led to the widespread belief that anti-lymphocytic sera could selectively eliminate the thymus-derived lymphoid cell populations. As a result these reagents have been frequently used as selective 'T' cell depletors in a number of immunological experiments of theoretical and practical importance.

In order to confirm and extend that anti-lymphocytic antibodies could suppress humoral immune responses and also whether this reagent exerted its immunosuppressive effect on humoral responses entirely via thymic cell population or whether other lymphoid cells were also implicated, the immunosuppressive effects of anti-lymphocytic antibodies were studied. The choice of antigens against which the immune responses have been studied was made on the current knowledge of cellular involvement in the immune response against these antigens. These included sheep erythrocytes, pneumococcal polysaccharide and bovine serum albumin.

It is now well established that to achieve an optimal immune response to sheep erythrocytes, it is essential that the animal in which this antigen is administered should have both the thymus-derived 'T' cell population and the antibody producing 'B' cell population (Miller, Mitchell and Weiss, 1967; Mitchell and Miller, 1968).

It is also known that thymus-derived cells do not secrete any antibody (Davies, Leuchars, Wallis, Marchant and Elliot, 1967), although they may produce and exhibit small amounts of antibody on their surface (Greaves, 1970; Bankhurst, Warner and Sprent, 1971; Marchalonis, Cone and Atwell, 1972). Furthermore, it has been shown that the antibody producing cells belong to a population of cells which originate from the bone marrow and develop to maturity independent of the thymic influence (Miller and Mitchell, 1968).

The other antigen pneumococcal polysaccharide which was employed in a large section of these studies was chosen for its ability to elicit an immune response at least in CBA mice without the apparent participation of thymus-derived cells (Humphrey, Parrott and deSousa, 1964; Davies, Carter, Leuchars, Wallis and Dietrich, 1970; Howard, Christie, Courtenay, Leuchars and Davies, 1971).

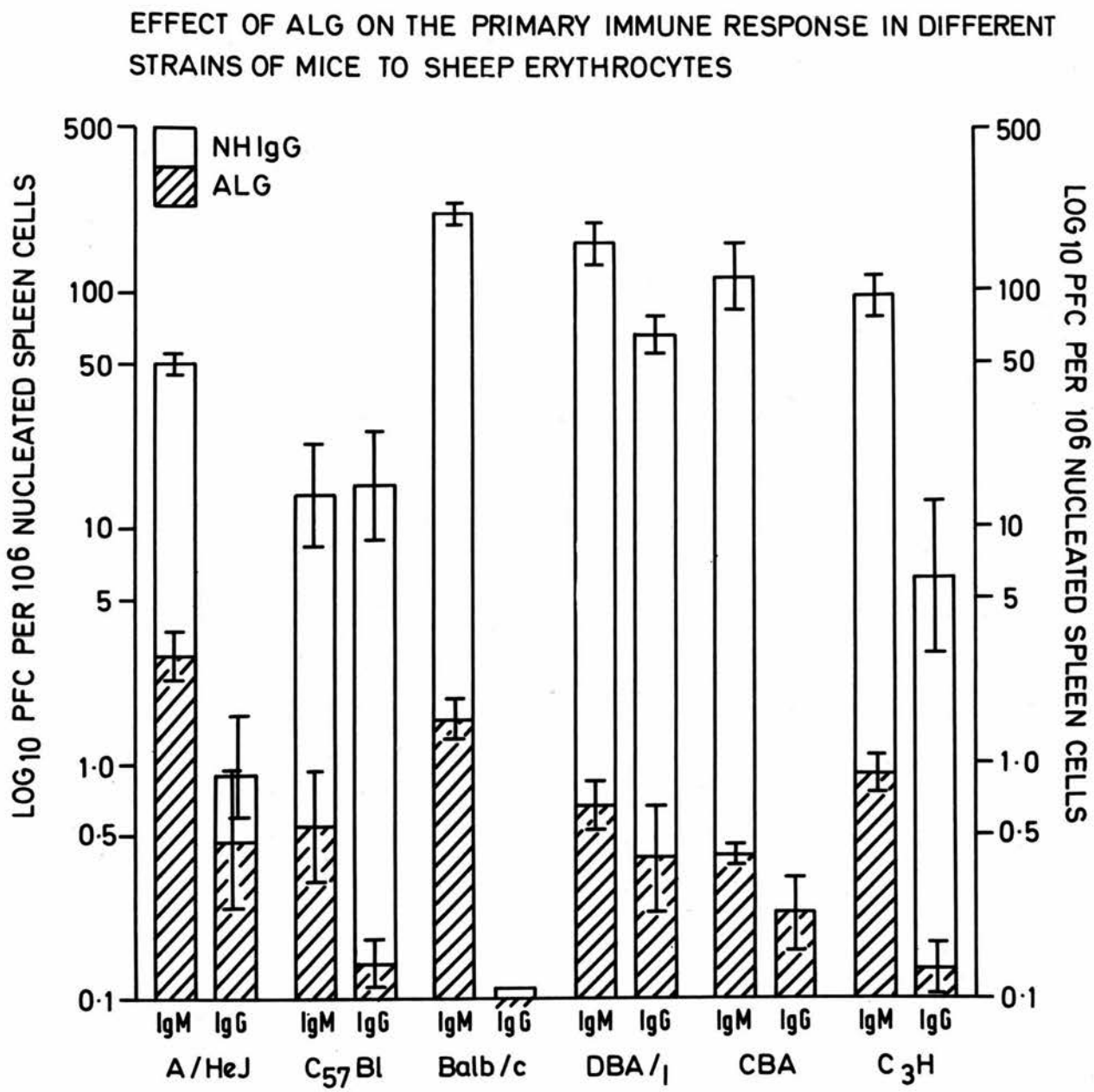
In addition the effect of ALG on the immune response against BSA was also studied in a limited number of experiments. This was to establish the effect of this reagent on the immune response of another antigen which requires the participation of the thymic lymphoid cell population (Taylor, 1969).

Apart from studying the effect of ALG on the immune response to several antigens, it was also felt necessary to study this in several strains of mice. This was based

on the strain variations observed in both rats and mice in the responsiveness to different antigens as well as in the effectiveness of ALG to suppress the immune response to certain antigens (James, Pullar and James, 1969; James and Milne, 1972).

From the results of experiments with sheep erythrocytes it is obvious that ALG 1d was adequately effective in suppressing the primary IgM (direct PFC) response to an optimal dose ($3 \times 10^8$) SRBC in all mouse strains tested (Figure 11 and Table 7). This is consistent with the previous findings reported by several workers (Barth and Southworth, 1968; Baum, Lieberman and Frenkel, 1969; Argyris and Plotkin, 1970). However, the effect of this ALG preparation on the primary IgG (developed PFC) response varied from strain to strain. In strains of mice where the control groups gave a measurable IgG response (C57Bl, DBA/1 and C3H in Table 7 and Figure 11), the response in ALG treated animals was significantly suppressed ($P<0.001$ in Figure 11). It should be noted that the differences between the response of NHIgG- and ALG-treated groups (i.e. the suppression achieved by ALG treatment) was generally more apparent when the PFC per $10^6$ cells (as opposed to PFC per spleen) were considered. This was due to a gross increase in the number of splenic nucleated cells following ALG treatment. Similar hyperplasia of the spleens in ALG-treated animals has been reported by

FIGURE 11


EFFECT OF ALG ON THE PRIMARY IMMUNE RESPONSE IN DIFFERENT STRAINS OF MICE TO SHEEP ERYTHROCYTES
NH IgG
ALG
LOG10 PFC PER 10⁶ NUCLEATED SPLEEN CELLS
500
100
50
10
5
1·0
0·5
0·1
IgM IgG
A/HeJ
C57 Bl
Balb/c
DBA/1
CBA
C3H

various other workers (Taub and Lance, 1968; Denman and Frenkel, 1968b; Marshall and Knight, 1969; Simpson and Nehlsen, 1970; Rodriguez-Paradisi, Thierfelder, Götze, Eulitz and Beil, 1970).

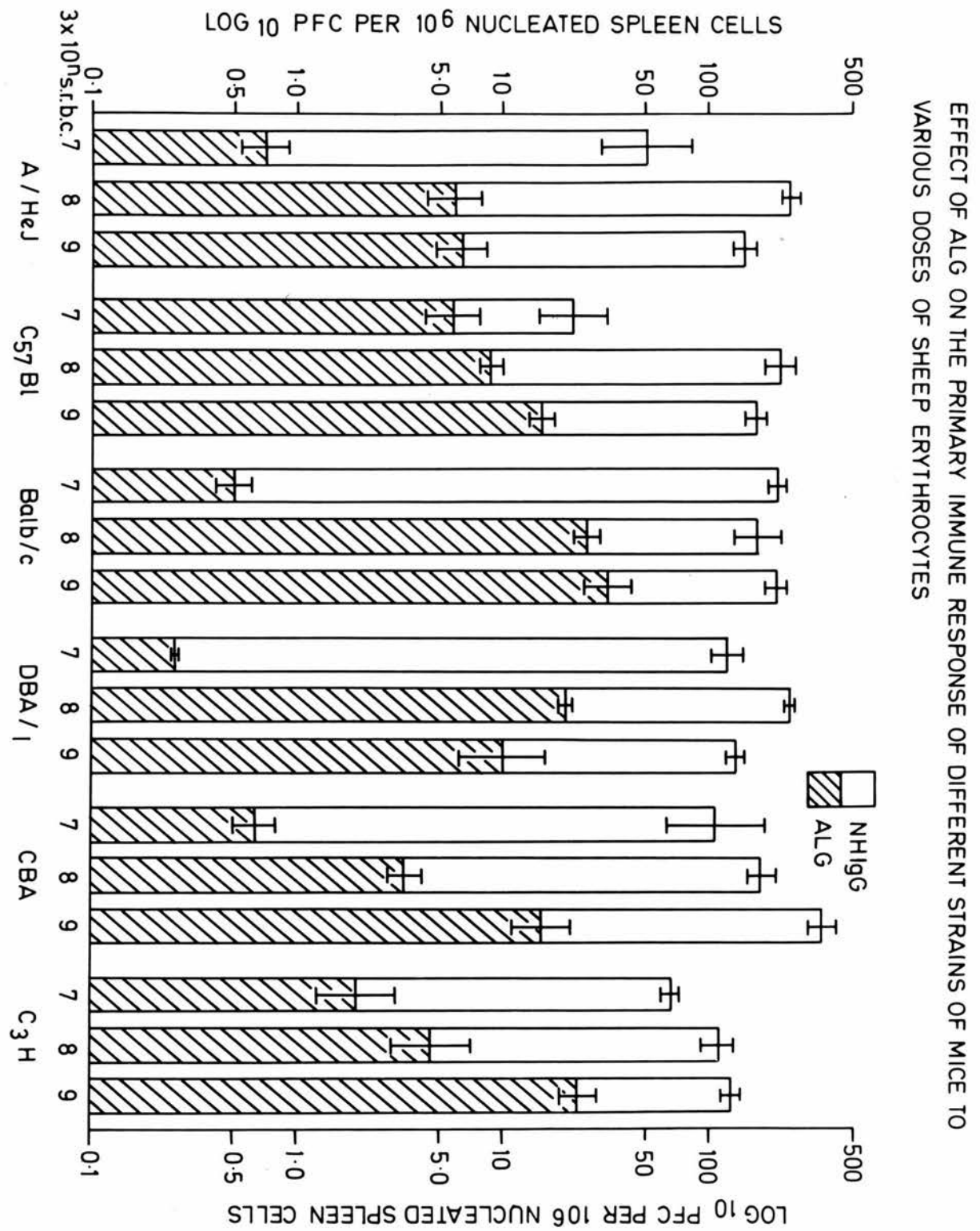
In contrast, in those strains of mice in which there was very little or no measurable IgG response in the NHIgG-treated groups, ALG treatment exerted an adjuvant-like effect (A/HeJ and CBA in Table 7). However, this augmentation by ALG was only significant in CBA mice (Table 7). This augmentation in the immune response to SRBC may be due to variations in the relative amounts of individual IgG subclasses evoked following the antigenic challenge. In this respect it is relevant to recall the findings of Anderson and her colleagues who reported a similar enhancement by ALG of the $IgG_1$ and $IgG_{2a}$ anti-SRBC response in CBA mice (Anderson, Dresser, Iverson, Lance, Wortis and Zebra, 1972). It is possible that the primary response to SRBC in certain strains, e.g. CBA and A/HeJ, are mainly in $IgG_1$ and $IgG_{2a}$ classes. The results of experiments to further investigate the effect of ALG on various immunoglobulin classes and subclasses will be discussed later.

Further experiments were designed to investigate the effect of ALG on the immune response to increasing doses of antigen. It has been reported that an increase in the dose of antigen could overcome the immunosuppressive

effects of ALG treatment (Lance, 1970a,b; Argyris and Plotkin, 1970). It could be postulated that ALG failed to suppress the immune response to higher antigen doses because an increasing dose of antigen could by-pass the need for the thymic cell participation in certain immune responses (Sinclair and Elliott, 1968; Taylor and Wortis, 1968; Playfair and Purves, 1971). However, contrary to the findings of Lance (1970a,b) and Argyris and Plotkin (1970) the present results indicate that increasing the dose of antigen ($3 \times 10^7$ to $3 \times 10^9$ SRBC) did not completely overcome the immunosuppressive effects of the ALG in any of the mouse strains tested (Figure 12). This may be due to difference in ALG treatment schedule or properties of the antibody preparation itself. However, it is appreciated that antigen doses in excess of $3 \times 10^9$ SRBC might overcome the immunosuppressive effects of the ALG treatment. Indeed there is a suggestion that the degree of suppression in animals given the highest antigen dose ($3 \times 10^9$ SRBC) is somewhat lower than that achieved in animals given the lower dose ($3 \times 10^7$ SRBC; Figure 12).

Several authors have reported results indicating the inability of ALG to suppress the secondary immune response of either rats or mice to sheep erythrocytes (James and Anderson, 1967; Lance, 1968a; Monaco, Wood and Russell, 1965) and to BSA (James and Jubb, 1967; James and Anderson, 1967; Lance, 1968a, 1970b; James and Milne,

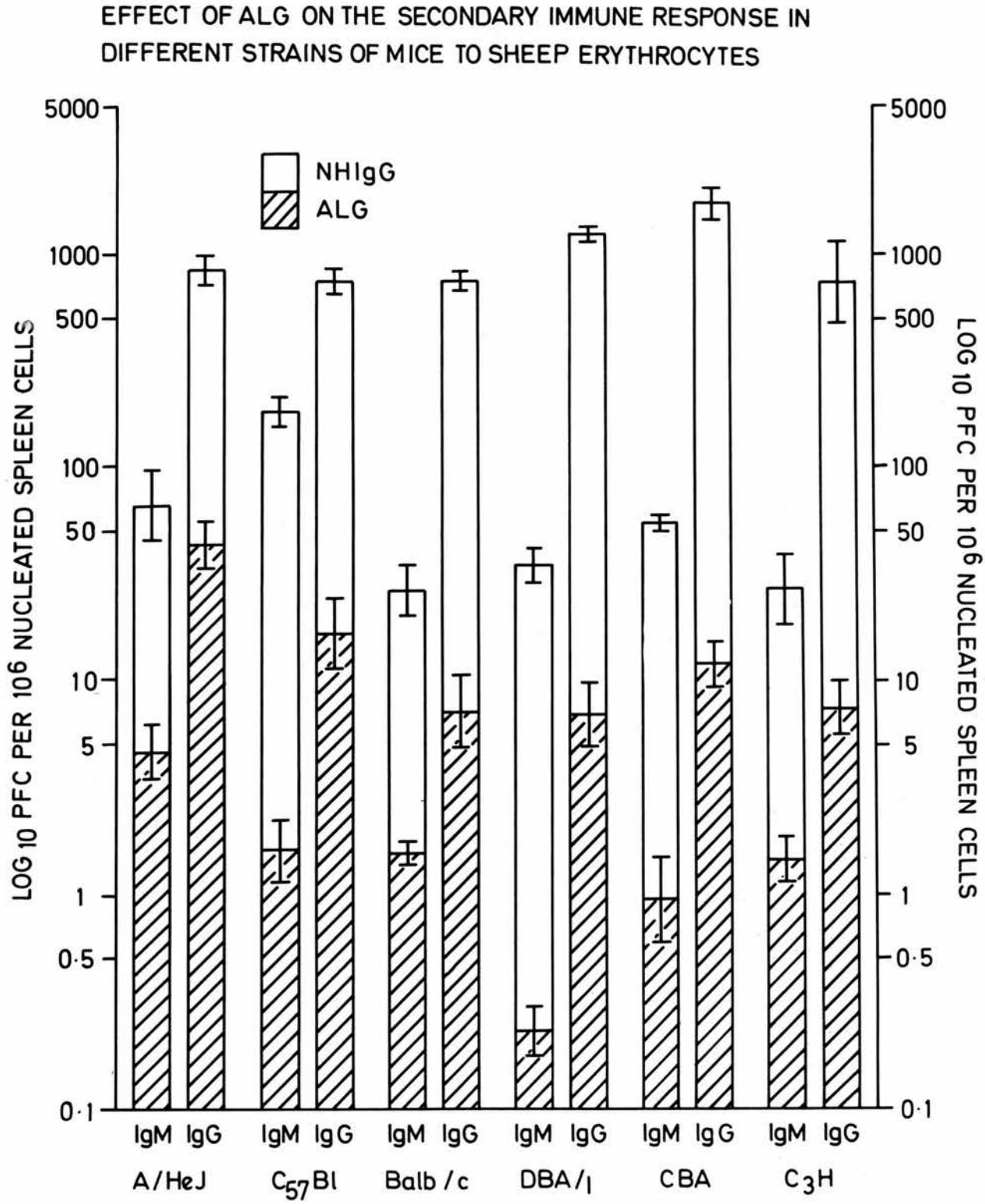
FIGURE 12


EFFECT OF ALG ON THE PRIMARY IMMUNE RESPONSE OF DIFFERENT STRAINS OF MICE TO VARIOUS DOSES OF SHEEP ERYTHROCYTES
LOG 10 PFC PER 10 6 NUCLEATED SPLEEN CELLS
3x10n s.r.b.c.
0·1
0·5
1·0
5·0
10
50
100
500
7
8
9
A / HeJ
C57 Bl
Balb/c
DBA/1
CBA
C3H
NHIgG
ALG

1972). In contrast to these reports, the ALG used in this series of experiments was able to suppress the secondary immune response to SRBC in all the mouse strains tested (Figure 13). However these findings are in general agreement with the earlier observations made by Barth and Southworth (1968) with SRBC, those of Marshall and Knight (1969) with BSA, and those of Lance (1970b) with Salmonella typhi 'H' antigen. These contrasting results could perhaps be explained on the basis of species variations, differences in the properties of ALG preparations used and the experimental models employed.

It is interesting to note in Figure 13 that the secondary response in A/HeJ mice was least susceptible to suppression by ALG and that this was most evident with respect to the IgG response. It should also be recalled that it was in A/HeJ mice that ALG treatment potentiated the secondary $IgG_{2a}$ response against sheep erythrocytes (Table 19) and failed to suppress the primary IgG response to this antigen (Table 7). This may also form the basis for explaining the inability of ALG to suppress the secondary response to alum BSA in the hands of various people (James and Jubb, 1967; Lance, 1968a, 1970a,b). On these occasions the antigen may have been stimulating the preferential production of antibody in certain immunoglobulin subclasses which are not readily suppressed (or indeed may be enhanced) by ALG treatment. In this connection, the recent findings

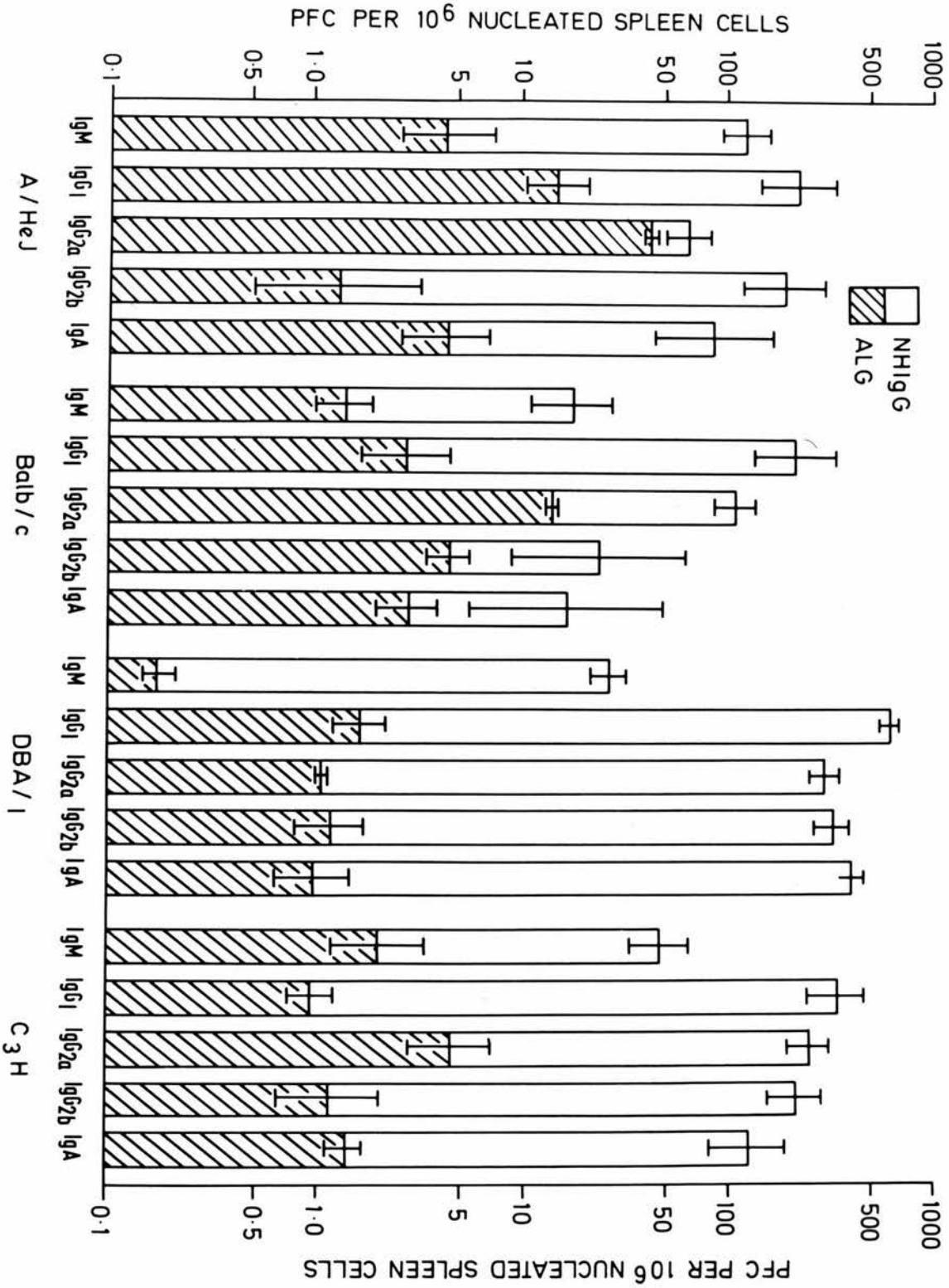
FIGURE 13


EFFECT OF ALG ON THE SECONDARY IMMUNE RESPONSE IN DIFFERENT STRAINS OF MICE TO SHEEP ERYTHROCYTES
NHIgG
ALG
LOG 10 PFC PER 10⁶ NUCLEATED SPLEEN CELLS
5000
1000
500
100
50
10
5
1
0·5
0·1
IgM IgG
A/HeJ
C57Bl
Balb/c
DBA/1
CBA
C3H

of Torrigiani (1972a) which indicated that the antibody response to alum-precipitated human serum albumin was mainly localized in $IgG_1$ and $IgG_{2a}$ subclasses and those of Anderson and her colleagues suggesting the augmentation of anti-SRBC response in these very IgG subclasses by ALG treatment may be of significance (Anderson, Dresser, Iverson, Lance, Wortis and Zebra, 1972). Also, it is possible that the utilization of different adjuvants might significantly influence the distribution of antibodies among the various immunoglobulin classes and subclasses. In this connection it is interesting to note that adjuvants can preferentially stimulate the production of antibodies in specific immunoglobulin classes and subclasses in both the mouse (Coe, 1966; Warner, Val and Ovary, 1968), and the guinea pig (Benacerraf, Ovary, Bloch and Franklin, 1963; White, Jenkins and Wilkinson, 1963).

In order to investigate the effect of ALG on classes or subclasses of antibody produced following antigen stimulation, the secondary immune response of different mouse strains to SRBC has been studied. Due to the limited amount of anti-immunoglobulin sera available, these studies were performed in only four mouse strains. The results of these studies clearly indicate that the ALG used in these studies (ALG 1d) successfully suppressed the IgM and $IgG_1$ responses in all four mouse strains tested (Figure 14 and Table 19). The $IgG_{2a}$ response was suppressed

FIGURE 14


EFFECT OF ALG ON THE SECONDARY IMMUNE RESPONSE OF DIFFERENT STRAINS OF MICE AGAINST SHEEP ERYTHROCYTES IN DIFFERENT IMMUNOGLOBULIN CLASSES
PFC PER 10^6 NUCLEATED SPLEEN CELLS
0·1
0·5
1·0
5
10
50
100
500
1000
NH IgG
ALG
IgM IgG1 IgG2a IgG2b IgA
A/HeJ
Balb/c
DBA/1
C3H

in all mouse strains except A/HeJ mice, whereas the $IgG_{2b}$ and IgA responses were not significantly suppressed in Balb/c mice. The latter seemed to be due to large variations in the responsiveness of individual Balb/c mice in these immunoglobulin classes (3-265 $IgG_{2a}$ and 1-258 IgG PFC per $10^6$ cells). However, in DBA/1 and $C_3H$ mice the ALG treatment suppressed antibody formation in all immunoglobulin classes and subclasses.

Several explanations can be offered for the differences between the results described here and those reported by Anderson and others (Anderson, Dresser, Iverson, Lance, Wortis and Zebra, 1972). Firstly, the results reported by Anderson and others were from studies on a late primary immune response. It is very likely that $IgG_1$ and $IgG_{2a}$ responses were the first to recover in the ALG-treated mice. Furthermore, since the ALG treatment had caused a suppression of anti-SRBC response in other immunoglobulin classes, some of which may have regulatory effects on $IgG_1$ and $IgG_{2a}$ antibodies, these responses may have overshot the responses in control mice not treated with ALG. Such a control of immune responses by antibodies have been demonstrated by various workers and these observations have been reviewed by Uhr and Moller (1968). Other factors which may account for the discrepancy include the differences in the properties of ALG preparations used and the strains of mice studied.

The data on the circulating serum agglutinin and haemolysin responses in general confirmed those obtained by PFC assays. The ALG treatment of mice resulted in the suppression of both the haemagglutinin and haemolysin responses. In order to measure the 7S haemagglutinin and haemolysin responses in these experiments, the sera were treated with 2-mercaptoethanol. It has been shown that reduction of sera with 2-mercaptoethanol destroys the ability of 19S and 11S antibodies to cause agglutination and haemolysis (Fudenberg and Kunkel, 1957; Rockey and Kunkel, 1962). It is nevertheless appreciated that some 7S antibodies may be sensitive to 2-mercaptoethanol treatment (Adler, 1965b).

The results of agglutinin and haemolysin titres in 2-mercaptoethanol treated sera (Tables 13 and 14) indicated that increasing the dose of antigen ($3 \times 10^7$ - $3 \times 10^9$ SRBC) resulted in an increased amount of 2-mercaptoethanol-resistant (IgG) antibody. A relative increase in the amount of IgG antibody with an increasing dose of SRBC has previously been observed (Wortis, Dresser and Anderson, 1969; Sinclair, 1967). None of the animals treated with ALG showed detectable amounts of 2-mercaptoethanol-resistant haemagglutinating or haemolytic antibody on the fourth or fifth days after the primary challenge (Tables 8-11 and 13-14).

There was a much greater proportion of 2-mercaptoethanol-

resistant antibody following the secondary challenge than there was following the primary one. An increase in the production of IgG antibody in the secondary immune response has been previously reported (Adler, 1965a; Sinclair, 1967; Sell, Park and Nordin, 1970). Nevertheless, ALG treatment caused a significant suppression of both total and 2-mercapto-ethanol-resistant antibody responses (Figures 15 and 16). It should be noted that haemolysin responses were more readily suppressed than haemagglutinin responses. Also, ALG treatment suppressed the 2-mercaptoethanol-resistant antibody titres more readily than it did the 2-mercapto-ethanol sensitive ones. It is also apparent that the suppression of secondary circulating antibody responses was not as effective as the suppression of primary ones. In addition, the effectiveness of ALG to suppress the secondary PFC responses was more apparent than the suppression of secondary circulating antibody responses. This is in agreement with the findings of Barth and Southworth (1968) who showed that ALG treatment significantly suppressed both direct and indirect secondary PFC responses, but failed to suppress the secondary agglutinin responses.

Experiments on the recovery of the immune response to SRBC in mice treated with ALG indicate that the primary immune response of immunosuppressed mice did not recover for a very long time. Indeed, it is appreciated that these experiments do not indicate the exact time of

FIGURE 15

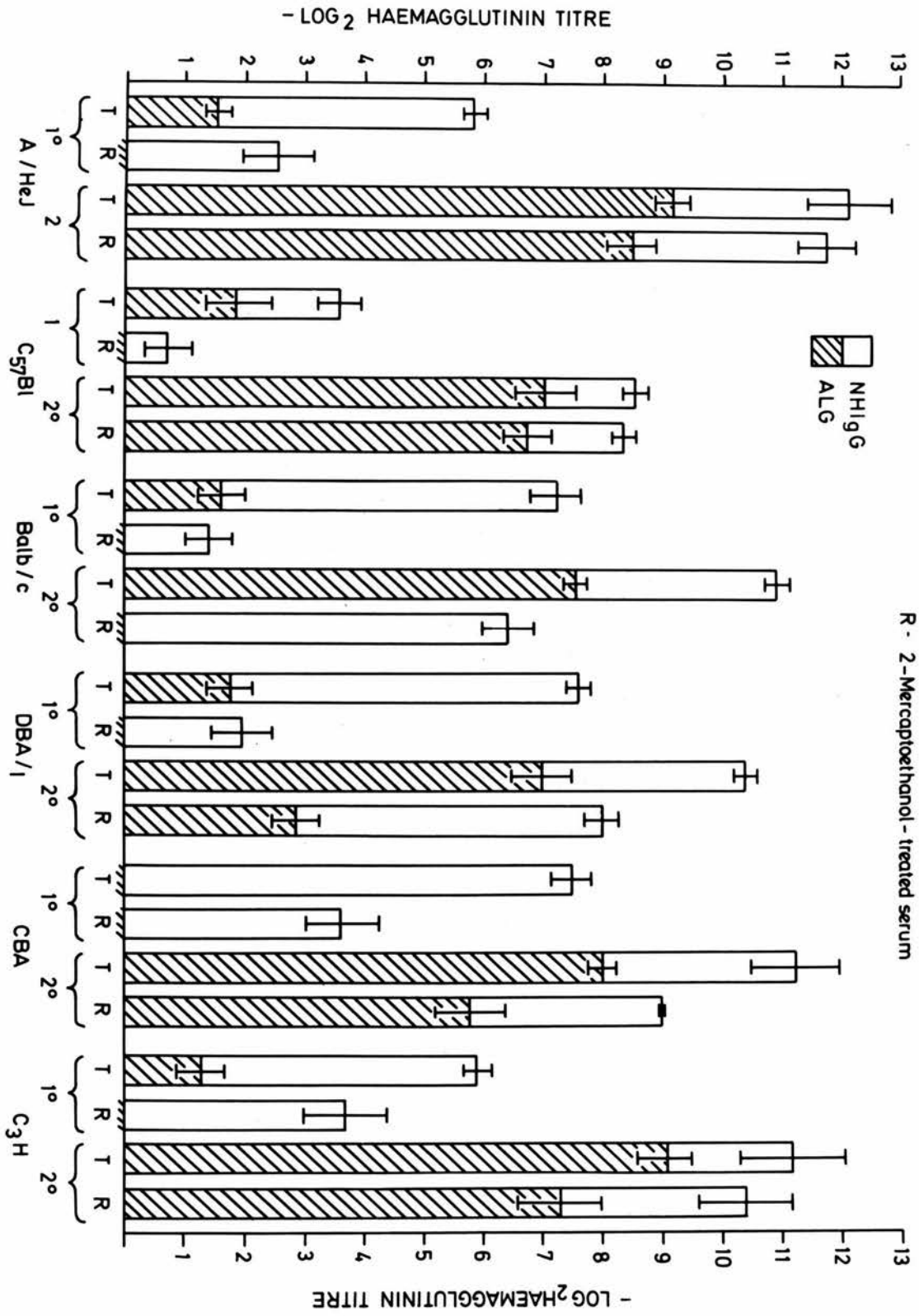
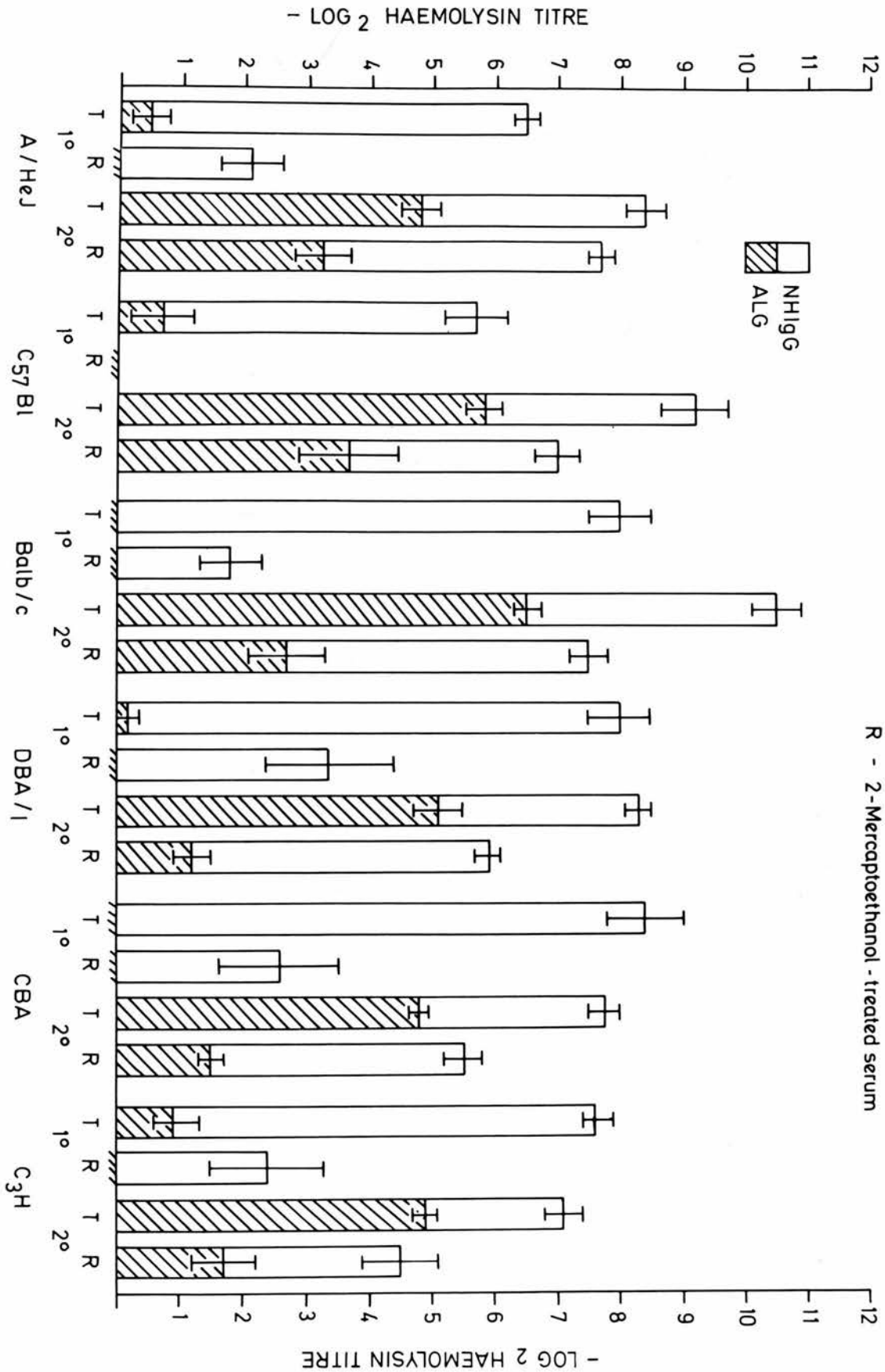

THE EFFECT OF ALG ON THE PRIMARY AND SECONDARY HAEMAGGLUTININ RESPONSE OF DIFFERENT MOUSE STRAINS AGAINST SHEEP ERYTHROCYTES
- LOG2 HAEMAGGLUTININ TITRE
NHIgG
ALG
T - Native serum
R - 2-Mercaptoethanol - treated serum
A/HeJ
C57Bl
Balb/c
DBA/1
CBA
C3H
1°
2°
T
R

FIGURE 16




THE EFFECT OF ALG ON THE PRIMARY AND THE SECONDARY HAEMOLYSIN RESPONSE OF DIFFERENT MOUSE STRAINS AGAINST SHEEP ERYTHROCYTES

recovery from a single regimen of ALG treatment. However, when these mice were rechallenged, they gave a secondary response no different from that of control mice (Figures 9 and 10). These observations are strictly in agreement with those recorded by other workers (Leuchars, Wallis and Davies, 1968; Baum, Liebermann and Frenkel, 1969).

The normal secondary response in ALG-treated animals clearly indicates that ALG treatment prior to the primary challenge, although it suppressed the primary antibody response, did not prevent the eventual sensitization of the recipient mice. This may imply that the ALG treatment suppressed the primary response not entirely by the elimination of SRBC antigen sensitive cells (of thymus or marrow origin), but it may also have caused the suppression by interfering with a step in the immune response following antigen recognition. The other alternative explanation for the preceeding findings may be that small amounts of antigen administered for the primary sensitization persisted for a long time and as the lymphoid cells (probably 'T' cells) recovered from the effects of ALG treatment, they became sensitized to the antigen. The latter hypothesis would be consistent with the earlier observations showing the inability of adult thymectomized animals to give a normal secondary response when treated with ALG prior to the primary sensitization with SRBC (Leuchars, Wallis and Davies, 1968). In this context it is interesting to

note that small amounts of SRBC antigen have been shown to persist in immunogenic form for up to 14 days after its administration (Britton, Wepsic and Moller, 1968). It should also be borne in mind that relatively small quantities of antigen are required to sensitize 'T' lymphocytes (Chiller, Habicht and Weigle, 1971; Playfair and Purves, 1971; Playfair, 1972).

The suppression of anti-SRBC response by the ALG preparation ld might be due to its effect on two major populations of lymphoid cells. It may have inactivated the thymus-derived lymphocytes which are essential for the induction of anti-SRBC response or have impaired the function of the precursors of antibody producing 'B' cells. It is appreciated that in addition to these two targets, ALG may be interfering with the functions of other cells involved in the immune response, e.g. macrophages. The indispensable nature of macrophages in the induction of immune response to different antigens is now well established (reviewed in the Introduction).

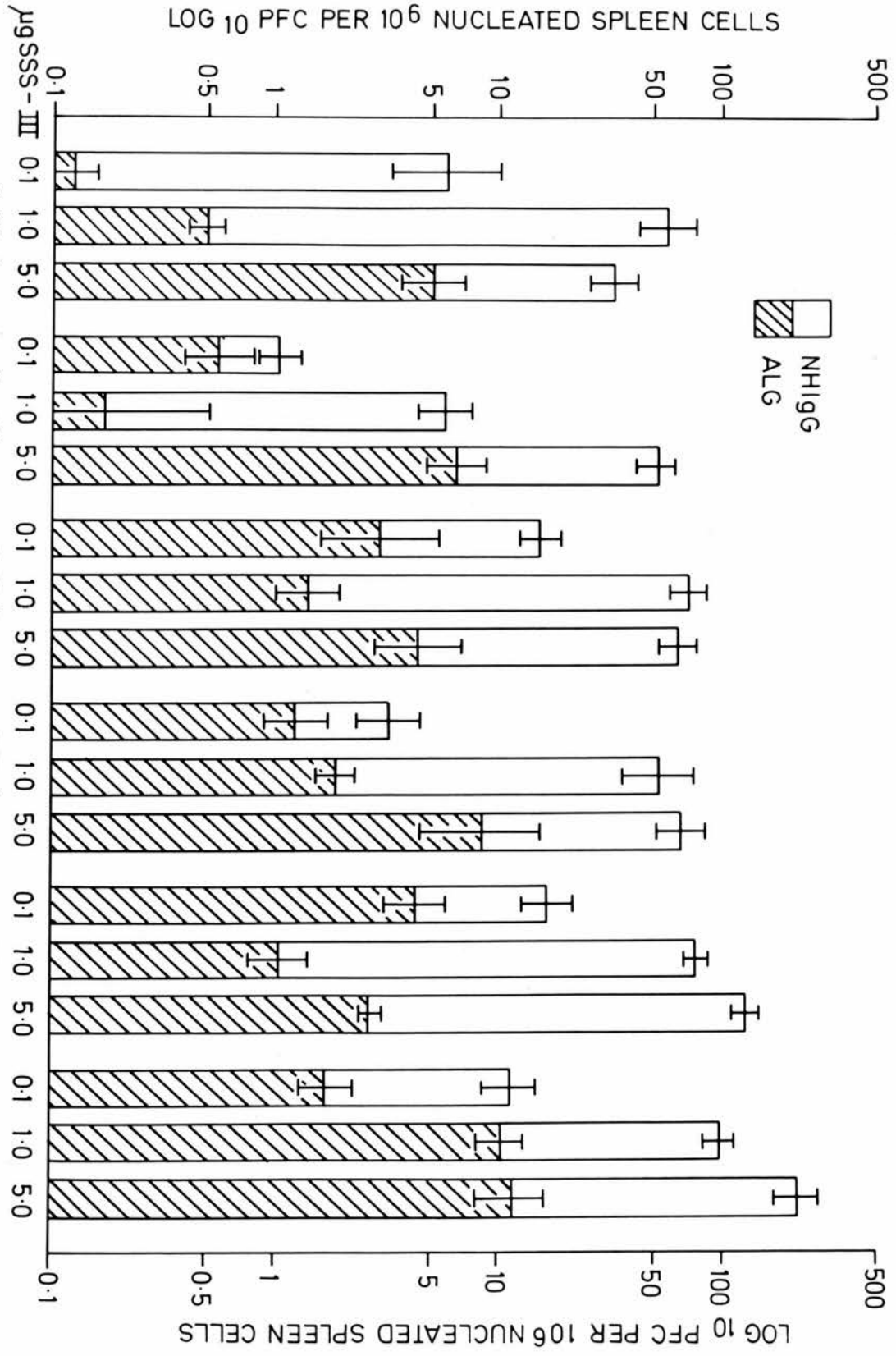
Antisera produced against macrophages have been shown to suppress the immune response to SRBC (Argyris and Plotkin, 1969, 1970) and other antigens (Isa, 1971; Schick, 1972). It has also been reported that antisera produced against lymphoid cells may strongly cross react with macrophages and related cells (Huber, Michlmayr and Fudenberg, 1969; Sheagren, Barth, Edelin and Malmgren, 1969; Woodruff,

Anderson and Abaza, 1966; DiLuzio and Pisano, 1970; Marsman, van der Hart and van Loghem, 1970). Furthermore, in vivo studies have shown that anti-lymphocytic antibodies may severely impair the clearance of macromolecular substances from the blood stream (Boak, 1968; Sheagren, Barth, Edelin and Malmgren, 1969; Grogan, 1969; Barth, Hunter, Southworth and Rabson, 1969). In addition a direct deficiency of macrophage function has been demonstrated following ALG treatment in rats where the suppression of anti-SRBC response could be reversed by the reconstruction of the ALG-treated animals with macrophages (Patterson, Pisano and DiLuzio, 1970).

In order to determine if the ALG preparation ld could act on the 'B' cell population in addition to 'T' cells the studies on its effect on the immune response to SSS-III were undertaken. As previously stated, it has been shown that thymus plays no role in the initiation of the immune response against this antigen (Humphrey, Parrott and DeSousa, 1964; Davies, Carter, Leuchars, Wallis and Dietrich, 1970; Howard, Christie, Courtenay, Leuchars and Davies, 1971).

The results of these experiments conclusively demonstrate that ALG ld was able to effectively suppress the immune response to SSS-III in a variety of mouse strains (Tables 24-26; Figure 17). These results confirmed the finding of James and Milne (1971) with

FIGURE 17


EFFECT OF ALG ON THE IMMUNE RESPONSE OF DIFFERENT STRAINS OF MICE TO VARIOUS DOSES OF PNEUMOCOCCAL POLYSACCHARIDE (SSS-III)
LOG 10 PFC PER 10⁶ NUCLEATED SPLEEN CELLS
μg SSS-III
0·1
0·5
1
5
10
50
100
500
NHIgG
ALG
A/HeJ
C57Bl
Balb/c
DBA/1
CBA
C3H
1·0
5·0

this antigen, and those of Allen, Freedman and Mills (1969) with E. coli lipopolysaccharide (LPS); the latter being another thymus independent antigen (Anderson and Blomgren, 1971; Möller and Michael, 1971). However, they are completely in contrast with the findings initially reported by Baker and his colleagues with SSS-III antigen in Balb/c mice (Baker, Stashak and Amsbaugh, 1970; Baker, Stashak, Amsbaugh, Prescott and Barth, 1970; Barth, Baker, Stashak and Amsbaugh, 1971), and those of Kerbel and Eidinger (1971) with polyvinyl pyrrolidone, and Veit and Michael (1972) with E. coli LPS.

It is also apparent from the results recorded in Figure 17 that the immunosuppressive capacity of ALG is not completely overcome by increasing the quantity of the antigen administered (at least in the dose range used in these experiments). Nevertheless the degree of suppression achieved by ALG seemed to be less marked in mice immunized with the higher antigen dose (5 μg.). The inability of the higher antigen dose to completely overcome the immunosuppressive capacity of ALG is once again in contrast with the findings with other antigens (Lance, 1970a,b; Argyris and Plotkin, 1970).

There are a number of possible explanations for the difference between these results and those of Baker and his colleagues. While at one time it was thought that one contributing factor might have been the mouse strains

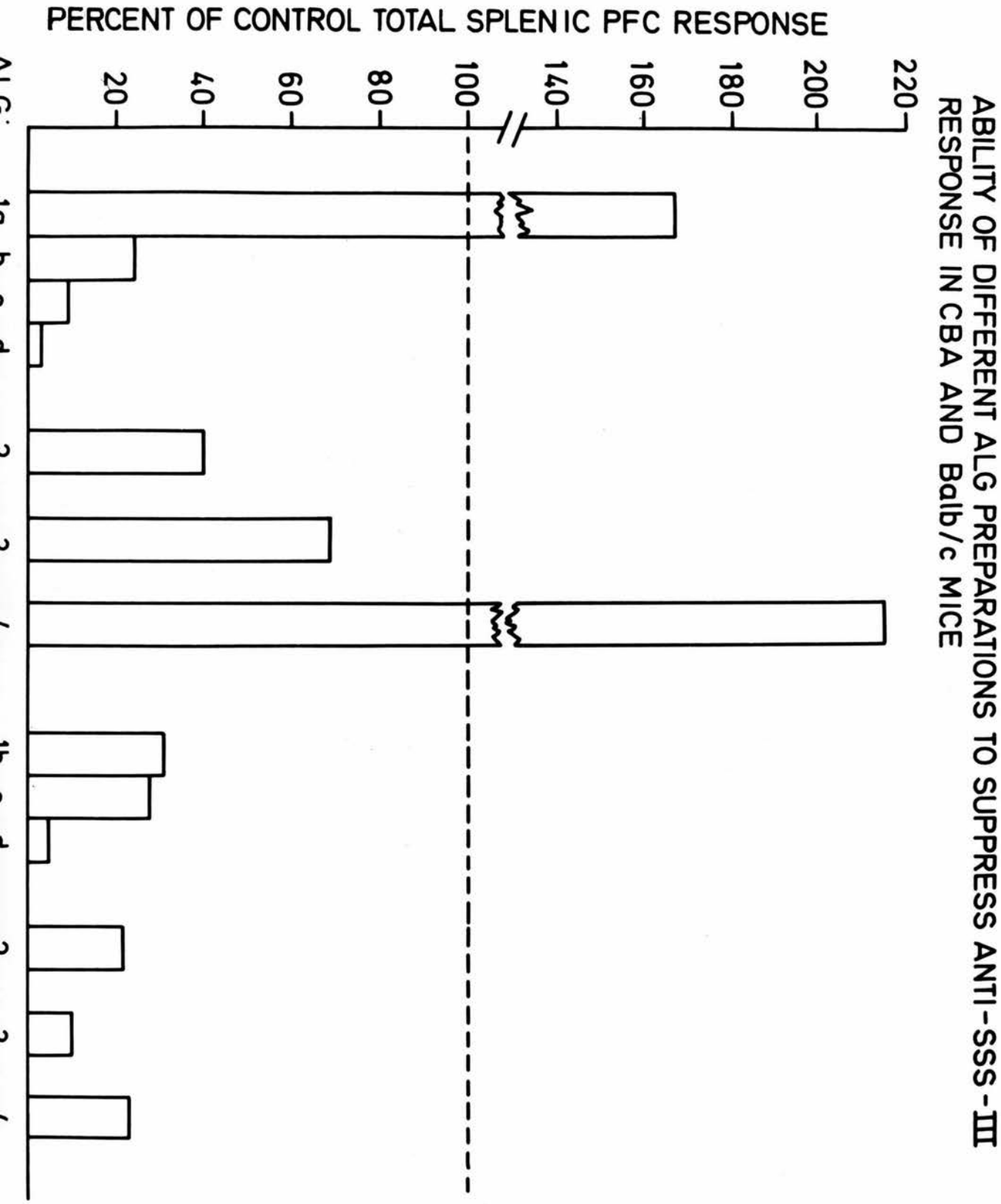
used (James and Milne, 1972), the present results rule out this possibility, for ALG ld suppressed the anti-SSS-III response in Balb/c mice. It should be recalled that the potentiation of anti-SSS-III response was observed in this very mouse strain (Baker, Barth, Stashak and Amsbaugh, 1970; Baker, Stashak, Amsbaugh, Prescott and Barth, 1970; Barth, Baker, Stashak and Amsbaugh, 1971). Furthermore, it is unlikely that they are due to the differences in the procedures for measuring the immune response, since the specificity of the assay procedure employed in these studies was established by the inhibition of anti-SSS-III plaques with purified soluble antigen (Figure 8). This was also true of the procedures used by Baker and his colleagues (Baker, Prescott, Stashak and Amsbaugh, 1971). Another two possible factors will be discussed later. These are basic differences in the ALG treatment schedule used and in the intrinsic properties of the ALG preparations.

Due to the difference between the present results and those of Baker and his colleagues it seemed imperative to ascertain if the ability of preparation ld to suppress anti-SSS-III response was unique to this product or was a property shared by other antisera. In order to determine this, a number of other ALG preparations raised in other horses were tested. It could also be argued that ALG ld was so effective in suppressing anti-SSS-III response because it was a multipulse serum and hence it had lost

the ability to discriminate between 'T' and 'B' cells. It should be stressed that previous observations have indicated a loss of specificity in anti-lymphocytic sera as a result of hyperimmunization of the production animal (Sutthiwan, Shorter, Hallenbeck and Elveback, 1969; Jooste, Lance, Levey, Medawar, Ruskiewicz, Sharman and Taub, 1968). Consequently ALG preparations were isolated from antisera obtained from a single horse at various times throughout its immunization course and their effects on the immune response to SSS-III were tested.

It is readily apparent from the results that the ability of anti-lymphocytic antibody to suppress the thymus independent response against SSS-III is not a property singly unique to the ALG ld preparation widely used in these studies. Nevertheless, it is perhaps true to say that this product appears relatively more efficient at suppressing the immune response to SSS-III than the other ALG preparations tested (Figure 18). It also appears from the results that certain ALG preparations (e.g. preparation 4 in Figure 18) may cause slight augmentation in the total splenic PFC response to SSS-III. However, on no occasion did this potentiation approach that previously observed by other workers with this antigen (Baker, Barth, Stashak and Amsbaugh, 1970; Baker, Stashak, Amsbaugh, Prescott and Barth, 1970; Barth, Baker, Stashak and Amsbaugh, 1971). It should be stressed that other workers have failed to confirm

FIGURE 18


ABILITY OF DIFFERENT ALG PREPARATIONS TO SUPPRESS ANTI-SSS-III RESPONSE IN CBA AND Balb/c MICE
PERCENT OF CONTROL TOTAL SPLENIC PFC RESPONSE
20
40
60
80
100
140
160
180
200
220

the results of Baker and his colleagues with a number of thymus independent antigens (Allen, Friedman and Mills, 1969; James and Milne, 1972; Veit and Michael, 1972). Furthermore, if the potentiation of immune response to thymus independent antigens by ALG treatment were due to the elimination of a suppressor 'T' cell population, as has been postulated by Baker and his colleagues (Baker, Stashak, Amsbaugh, Prescott and Barth, 1970), a similar effect should be produced by the elimination of 'T' cells by neonatal thymectomy or by adult thymectomy and radiation. However, so far no such effect has been observed following these treatments (Howard, Christie, Courtenay, Leuchars and Davies, 1971; Andersson and Blomgren, 1971; Veit and Michael, 1972).

It can also be seen from the results that whenever the total PFC response was potentiated by ALG treatment, this could at least partly be attributed to the accompanying splenic hyperplasia. For, the PFC per $10^6$ nucleated spleen cells never significantly exceeded that observed in the normal IgG-treated controls. As previously emphasized an increase in plasma cell and other cell populations has been observed by previous investigators following ALG treatment (Taub and Lance, 1968; Denman and Frenkel, 1968b; Marshall and Knight, 1969; Simpson and Nehlsen, 1970; Rodriguez-Paradisi, Thierfelder, Gotze, Eulitz and Beil, 1971).

The ability of certain ALG preparations to suppress the immune response to SSS-III is undoubtedly influenced by the strain of animals in which they are tested (see Figure 18) although this difference was not noticeable with ALG 1d. In this respect it is interesting to note that the products tested were more efficient in Balb/c mice, the strain in which previous investigators found a significant enhancement of anti-SSS-III response following treatment with ALG (Baker, Barth, Stashak and Amsbaugh, 1970; Baker, Stashak, Amsbaugh, Prescott and Barth, 1970; Barth, Baker, Stashak and Amsbaugh, 1971). This further excludes the possibility that the differences in the results of the two studies could be due to the mouse strain used. It should be re-emphasized that similar strain specific effects following treatment with ALG have been observed in rats (James, Pullar and James, 1969) and mice (James and Milne, 1972). It is also interesting to note that the relative sensitivity of Balb/c mice to the immunosuppressive effects of ALG could not be attributed to the origin of thymocytes used for immunization as none of the preparations were raised by immunization with thymocytes from this mouse strain.

One of the factors most likely to explain the differences between the present results and those of Baker and his colleagues was the schedule of ALG treatment used. The results so far discussed were obtained from animals treated

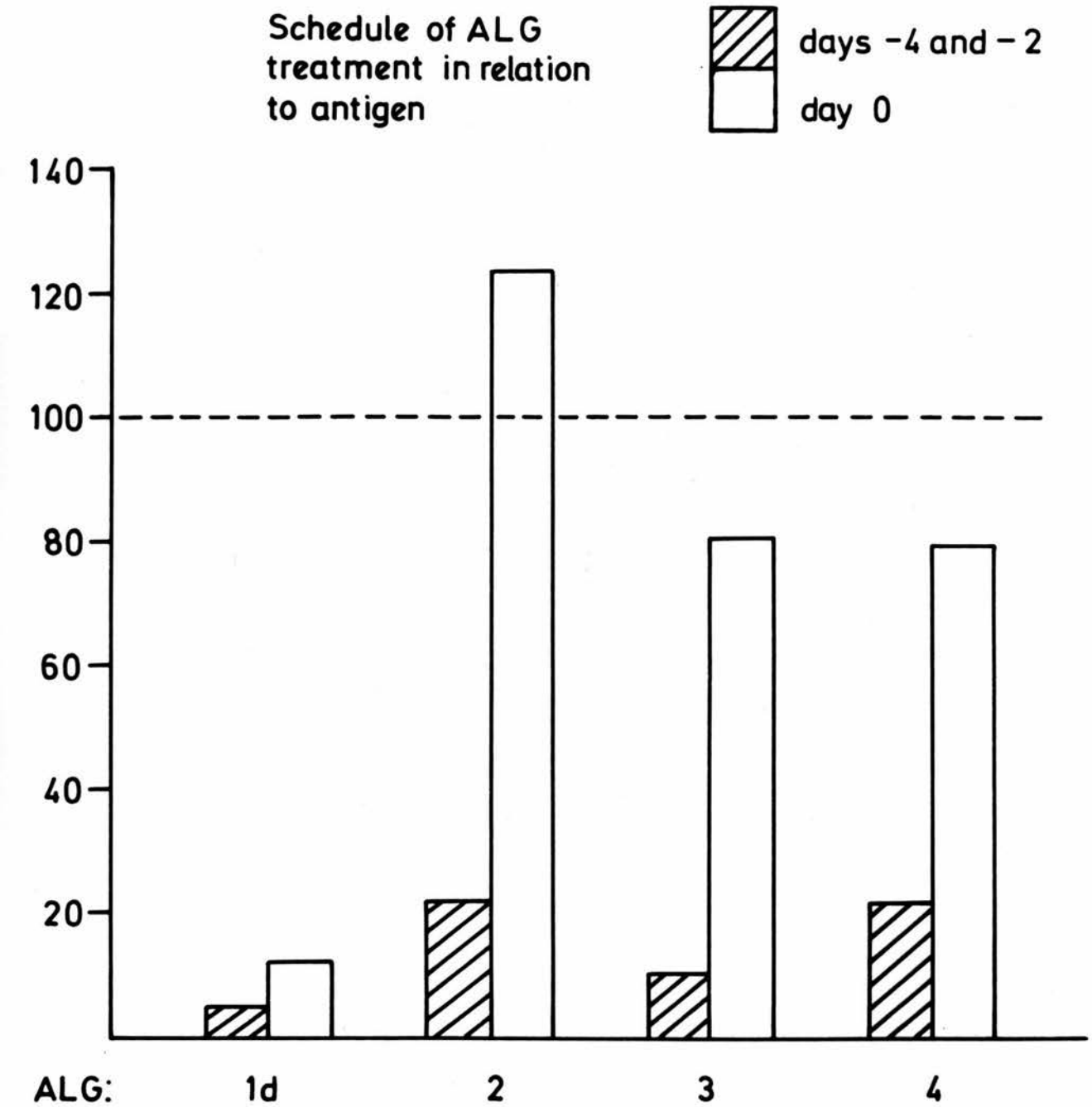
with ALG on days -4 and -2 prior to antigen administration. In contrast in the bulk of the studies by Baker and his colleagues the ALG was administered simultaneously with (or just before) the antigen (Barth, Baker, Stashak and Amsbaugh, 1971). In order to test this possibility the two schedules of ALG treatment were compared in Balb/c mice.

The results of these studies clearly indicate that the immune response to SSS-III is more readily suppressed if the ALG treatment is commenced some days prior to antigenic challenge (Figure 19). If the ALG treatment was delayed until just prior to antigenic challenge then it may be less effective (as in the case of preparation 1c) or without a significant effect (see preparations 2-4). A similar situation has been noted with respect to the immune response of rats to alum BSA (James, 1967) and mice to SRBC (Berenbaum, 1967; Baroni, Kimball, Ward and Wagar, 1969). While this might explain in part the differences between the results recorded here and those previously reported by Baker and his colleagues (Baker, Barth, Stashak and Amsbaugh, 1970; Baker, Stashak, Amsbaugh, Prescott and Barth, 1970; Barth, Baker, Stashak and Amsbaugh, 1971), it could not be the sole explanation, for, in no instance was there the marked potentiation observed by Baker and his colleagues.

As stated earlier, one possible explanation of these

FIGURE 19

EFFECT OF ALTERNATIVE SCHEDULE OF ALG TREATMENT ON THE IMMUSUPPRESSIVE ABILITY OF DIFFERENT ALG PREPARATIONS



discrepancies is unique differences in the various ALG preparations used. In order to determine if the effectiveness of the ALG was due to the multiple immunization schedule used in its production, the effect of ALG preparations isolated from sera obtained at various stages of immunization of the ALG donor were tested.

It is obvious from the results that the ability of ALG 1d to suppress anti-SSS-III response could not be merely due to hyperimmunization of the horse, for, the ALG preparation obtained after only two injections of thymocytes (1b) was already effective at suppressing anti-SSS-III response (see Figure 18). The ALG preparation 1a which did not suppress the immune response to SSS-III also failed to suppress the anti-SRBC response (Figure 21). In addition product 1d was more effective than products 2-4 which were produced by extremely chronic and/or prolonged immunization schedule (see Table 2). Furthermore, the inactive product described by previous authors (Baker, Barth, Stashak and Amsbaugh, 1970; Baker, Stashak, Amsbaugh, Prescott and Barth, 1970; Barth, Baker, Stashak and Amsbaugh, 1971) was produced by a fairly chronic immunization schedule (Barth, 1969). However, it is obvious that the immunosuppressive potency of ALG produced in a single horse did increase with repeated immunization (Figure 18). It should be stressed that none of the products employed in these studies were toxic <u>in vivo</u> after absorption with

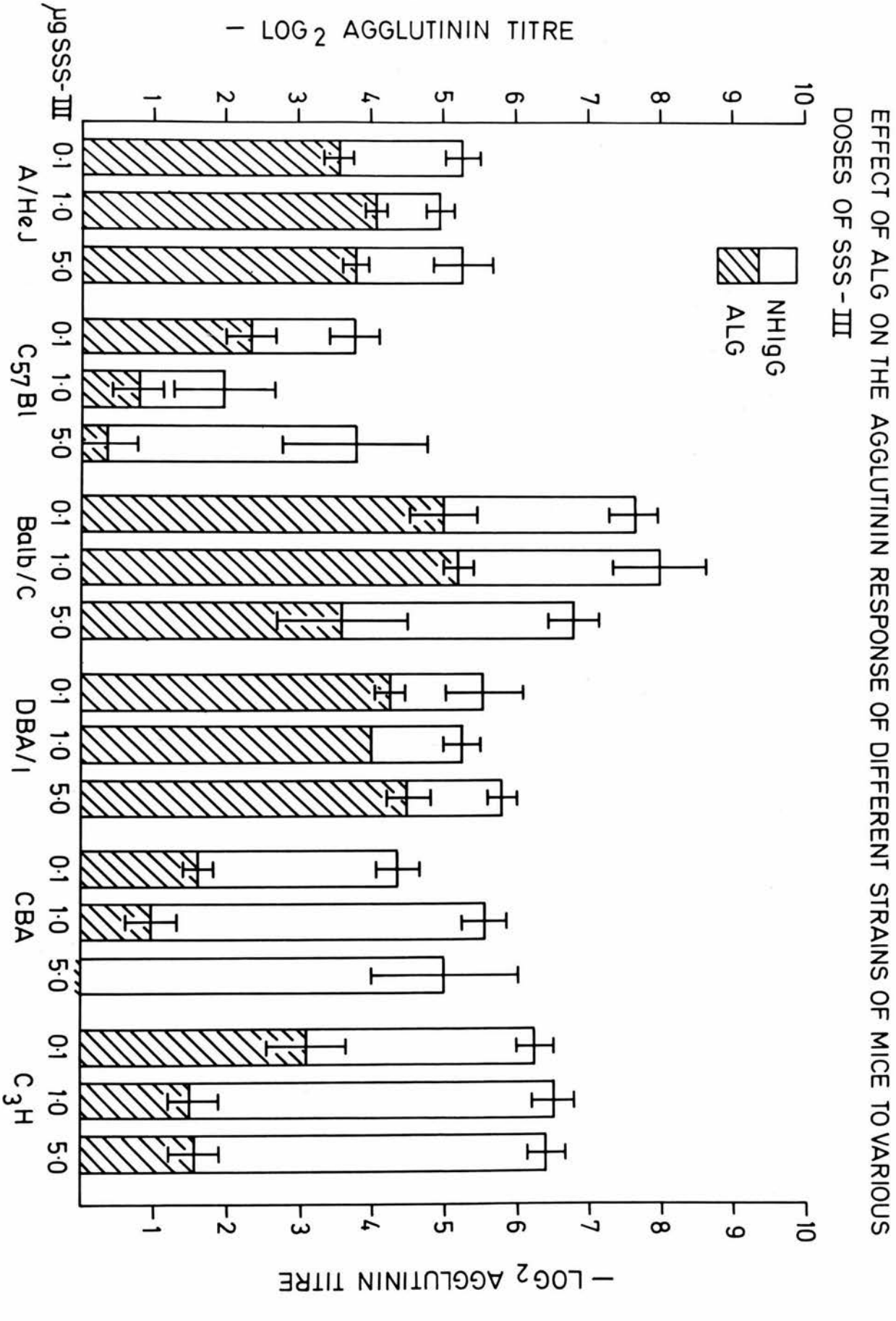
mouse erythrocytes.

In conclusion, the most likely explanation of the differences between these results and those of other workers (Baker, Barth, Stashak and Amsbaugh, 1970; Baker, Stashak, Amsbaugh, Prescott and Barth, 1970; Barth, Baker, Stashak and Amsbaugh, 1971; Kerbel and Eidinger, 1971) are differences in the ALG treatment schedule and the basic differences in the properties of the ALG preparations themselves. The latter differences may be determined by the nature of the antigen used in the production of anti-lymphocyte sera, the immunization schedule adopted, the mode of antigen administration (i.e. its incorporation into adjuvants) and the immune response of individual production animals. The fact that the antisera used in this study were produced against normal thymocytes in horses and the antiserum used by Baker and his colleagues was raised against methylcholanthrene-induced lymphoma cells in burro (Barth, 1969) may be significant. It is also interesting to note that antisera used in other studies where no suppression of thymus independent responses were noted were produced without adjuvant (Kerbel and Eidinger, 1971; Veit and Michael, 1972). However, it cannot explain the differences between the results reported here and those of Baker and his colleagues, for, they also used adjuvant in the production of their antiserum.

While most of the ALG preparations tested significantly suppressed the PFC response to SSS-III, their effect on circulating formation was less marked (Figure 20). This may be due to qualitative differences in the antibodies measured by these two techniques, e.g. immunoglobulin classes, binding affinities, etc. It should be pointed out that such differences have been recorded by certain investigators (Kearney and Halliday, 1970a,b).

Throughout these studies it was assumed that the immune response to type-III pneumococcal polysaccharide is completely thymus independent (B-cell process), at least as far as CBA mice are concerned and there exists a considerable amount of evidence to support this assumption (Davies, Carter, Leuchars, Wallis and Deitrich, 1970; Howard, Christie, Courtenay, Leuchars and Davies, 1971). Accepting that a similar situation is operative in other strains of mice, and there is data to suggest that this is so (Humphrey, Parrott and East, 1964; Manning, Reed and Jutila, 1972), one would conclude that ALG suppresses the immune response to this antigen by inactivating or interfering with the function of the thymus independent (B) lymphoid cell population. However, there is a body of evidence that anti-lymphocyte sera may react with lymphoid cells of extra-thymic origin. For example using colony forming unit assays several authors have showed that ALG could damage haemopoietic cells in the bone marrow <u>in vivo</u>

FIGURE 20


EFFECT OF ALG ON THE AGGLUTININ RESPONSE OF DIFFERENT STRAINS OF MICE TO VARIOUS DOSES OF SSS-III
— LOG2 AGGLUTININ TITRE
NHIgG
ALG
μg SSS-III
0·1
1·0
5·0
A/HeJ
C57Bl
Balb/C
DBA/1
CBA
C3H
1
2
3
4
5
6
7
8
9
10

(DeMeester and Anderson, 1968; DeMeester, Anderson and Shaffer, 1968; Nouza, Haskovcova and Nemec, 1971). In addition Jeejeebhoy and Singla (1972b) showed that the inability of ALG-treated rats to respond to SRBC was in part due to temporary damage or inactivation of bone marrow cells. Also, Andersson and her colleagues (1970) demonstrated that their ALG preparation could suppress anti-hapten response in an adoptive situation where the donors of the producer lymphoid cells were treated with ALG. It should be noted that the production of anti-hapten antibody is carried out by 'B' cells (Mitchison, 1971a). Furthermore, the recent observations of Janossy and Greaves (1972) who demonstrated that anti-thymocyte antibody preparations could interfere in vitro with either 'T' cell or 'B' cell responses or both provide additional support to the view that ALG can react with non-thymic lymphoid cells. Finally, there is abundant evidence that anti-lymphocyte sera may interfere with the function of macrophages (see earlier), a cell population which might be involved in the immune response against SSS-III (Aaskov and Halliday, 1971). It is possible that the effectiveness of certain ALG preparations (like ld in these studies) in suppressing both thymus dependent and thymus independent humoral immune responses may be due to their ability to interfere with the function of 'T' cells, 'B' cells and macrophages.

The results obtained from the preliminary cell reconstitution experiments suggest that the immunological injury caused by the ALG treatment may be repaired, at least partially, by either 'B' cells (Table 37, group D) or 'T' cells (Table 37, group C). The bearing of these findings on the thymus dependence of the SSS-III response is not clear at present. It should, however, be noted that recent evidence suggests that thymus cells may play a role in the immune response to SSS-III, as the response to this antigen in thymus-deprived mice has been shown to be less sustained than in reconstituted mice (Howard, Christie, Courtenay, Leuchars and Davies, 1971). In the experiments reported here, it is possible that reconstitution of anti-SSS-III response by thymocytes was achieved simply because these cells accelerated the regeneration or proliferation of 'B' cells in lymphocyte depleted mice. It should however be stressed that before any definite conclusions are drawn the reproducibility of these findings must be ascertained.

In order to establish that ALG preparations 1a-1c and 2-4 were comparable with the 1d preparation, their effect on the immune response against thymus dependent antigens, BSA and SRBC was examined. It is apparent that ALG preparations 2-4 also significantly suppressed these responses, although the degree of suppression varied from one preparation to the other (Figures 21 and 22). It is

also interesting to note that their ability to suppress thymus independent responses was not related to their effectiveness on thymus dependent responses (compare effect of ALG 2-4 in CBA mice in Figure 18 with that in Figures 21 and 22).

It is also apparent that ALG 1a effectively suppressed the immune response to BSA but did not suppress the IgM responses against SRBC and SSS-III. Furthermore all the ALG preparations were much more effective at suppressing IgG responses to SRBC than IgM responses (1a-1d and 2-4; Figure 21). It should also be stressed that ALG 1a suppressed the IgG anti-SRBC response but failed to significantly suppress the anti-SRBC IgM response (Figure 21). It should be pointed out that the anti-BSA response measured at three weeks after immunization would be expected to be mostly in the IgG immunoglobulin class. It may be relevant to mention here that Torrigiani (1972a,b) found that immune response to alum-precipitated human serum albumin at 3 weeks after immunization was mostly in the IgG class. Thus it appears that the ALG is generally more effective in suppressing immune responses of IgG immunoglobulin class. This is also supported by the data from the 2-mercaptoethanol sensitivity of sera obtained from control and ALG-treated animals (Figures 15 and 16).

The production of certain IgG subclasses is believed to be dependent on the participation of the thymus. This

FIGURE 21

ABILITY OF DIFFERENT ALG PREPARATIONS TO SUPPRESS THE PRIMARY ANTI - SRBC RESPONSE

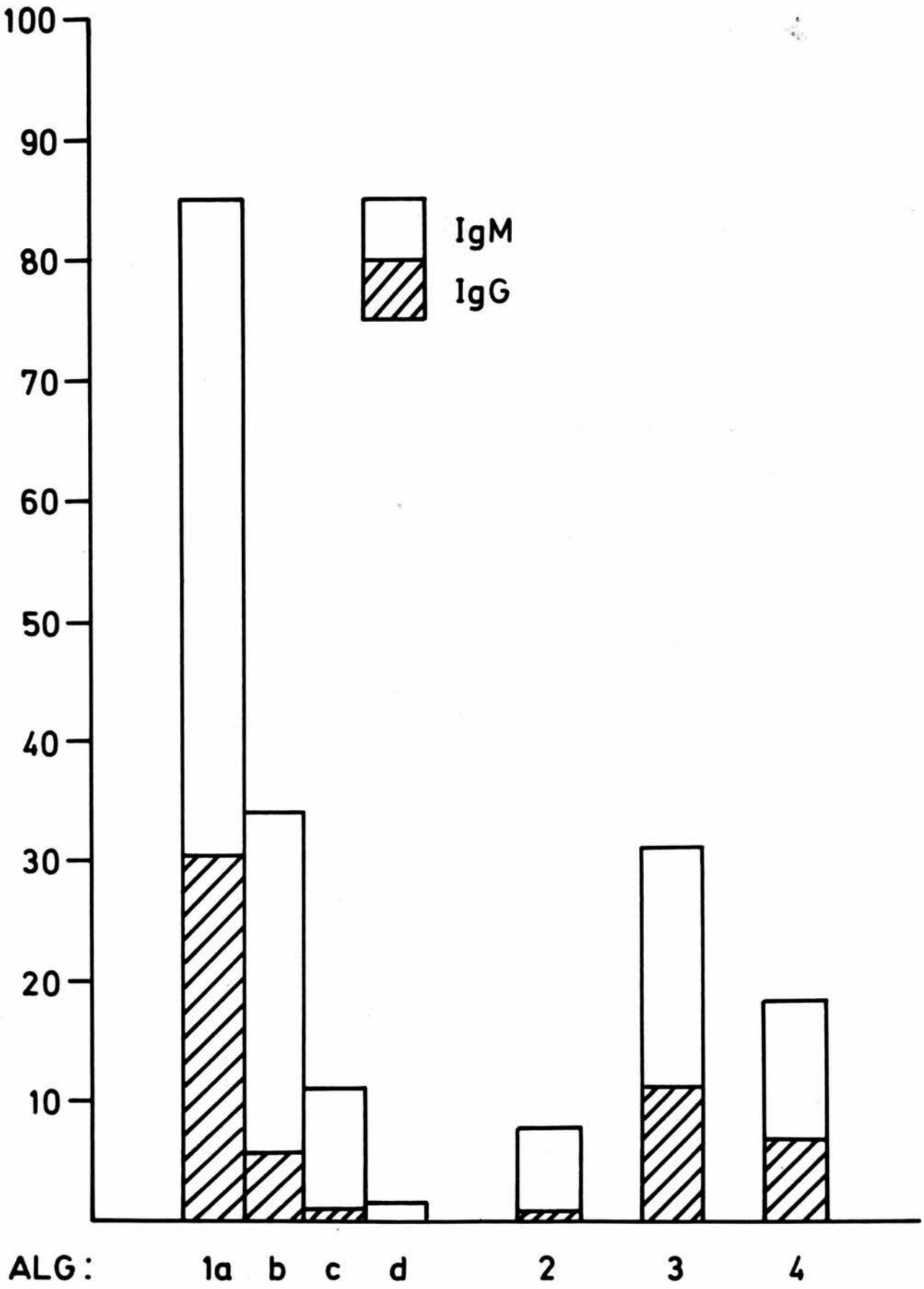
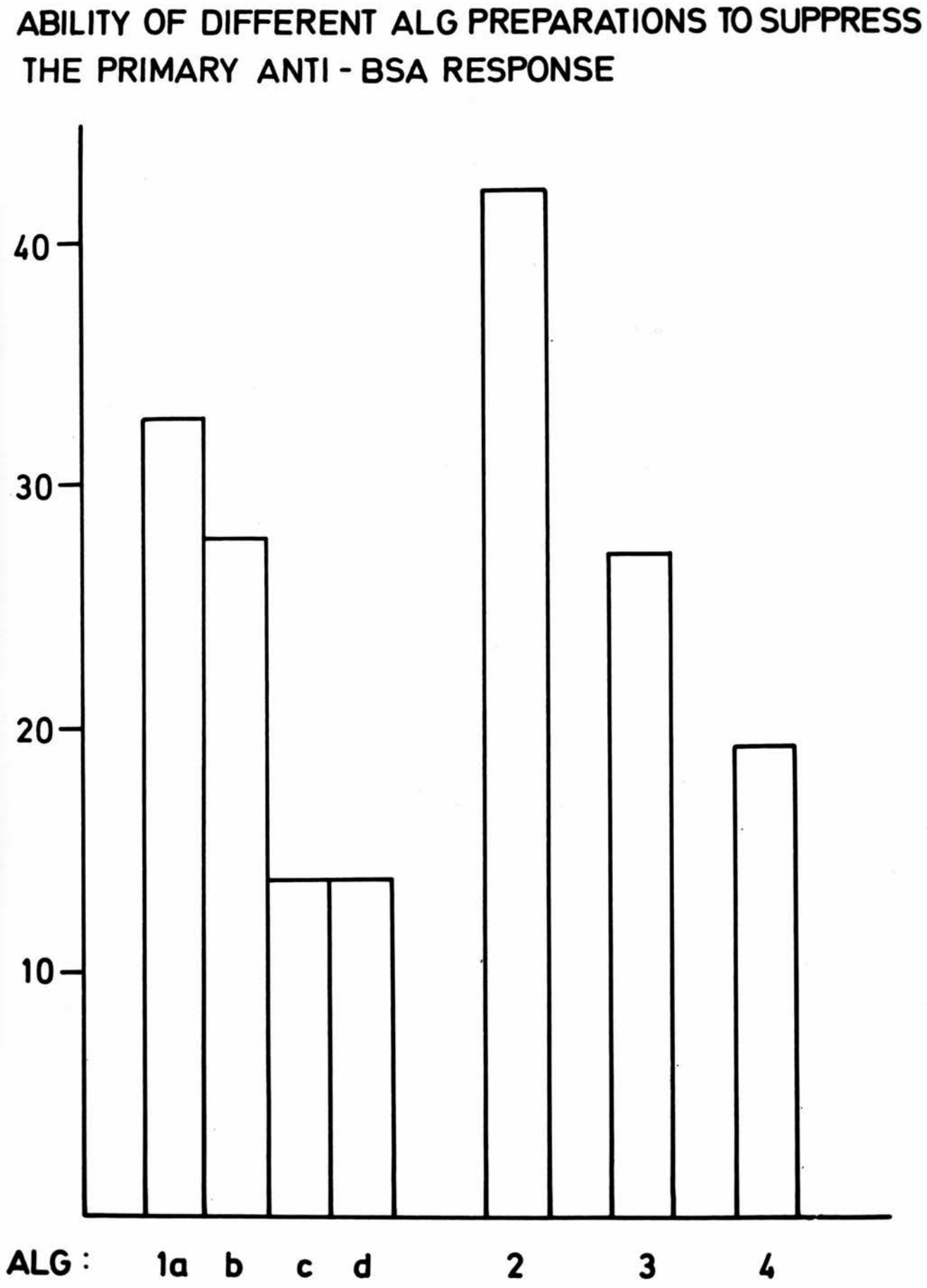


FIGURE 22


ABILITY OF DIFFERENT ALG PREPARATIONS TO SUPPRESS THE PRIMARY ANTI - BSA RESPONSE
40
30
20
10
ALG :
1a
b
c
d
2
3
4

view is based upon the relative deficiency of IgG in mice displaying thymic aplasia (Luzzati and Jacobson, 1972; Pritchard, Riddaway and Micklem, 1973) and in humans with thymic deficiency diseases (Peterson and Good, 1968; Siegal, Pernis and Kunkel, 1971). Similarly, mice experimentally made thymus-deficient show a decrease in the level of antibody response in certain IgG subclasses (Taylor and Wortis, 1968; Torrigiani, 1972b). It should also be mentioned that the most of the immune responses which are produced in the absence of thymic influence are mainly of the IgM type (Britton and Moller, 1968; Taylor and Wortis, 1968; Aird, 1971; Howard, Christie, Courtenay, Leuchars and Davies, 1971; Pritchard, Riddaway and Micklem, 1973). On this basis it will be reasonable to postulate that ALG can generally suppress thymus dependent responses more effectively and with greater ease than the thymus independent ones.

While the results of the studies reported here indicate that antisera capable of suppressing 'B' cell responses may be produced by modest immunization schedules, it might be possible to produce antisera capable of specifically inhibiting 'T' cell responses by the 2-pulse immunizing schedule of Levey and Medawar (1966a) which does not employ adjuvants. It should again be recalled that antisera used by authors who were not able to suppress the immune responses against thymus independent antigens were raised

by this procedure (Kerbel and Eidinger, 1971; Veit and Michael, 1972). However, an overall consideration of the data presented here clearly indicates that the use of anti-lymphocytic sera as selective depletors in immunological systems (a practice pursued in some laboratories) may on occasions give rise to erroneous conclusions. It is evident that before such antisera can be used for this purpose, their specificity must be carefully established. Their effect on the immune responses against thymus independent antigens may be one of the *in vivo* methods of some value in determining their specificity.

# CONCLUDING REMARKS

From the data presented in this thesis (see Tables 39 and 40), it can be concluded that most ALG preparations are capable of suppressing humoral immune responses against the thymus dependent antigens SRBC and BSA. In addition, a number of ALG preparations can also suppress the immune responses against thymus independent antigens (e.g. SSS-III). Thus, anti-lymphocytic sera may exert their immunosuppressive effects by interfering with the function of both 'T' and 'B' cells. However, the evidence presented does suggest that thymus dependent immune responses are more susceptible than the thymus independent ones. Furthermore, the action of anti-lymphocytic sera through macrophages remains a likely possibility.

It is also apparent that the immunosuppressive properties of ALG may be influenced to some extent by the dose of antigen administered and the strain of animals in which they are tested. In addition, the ability of ALG at suppressing humoral immune responses may largely depend upon the time of its administration in relation to the antigenic challenge.

Finally, the results indicate that individual ALG preparations may differ greatly from each other in their immunosuppressive properties even though they are raised in the same species by almost identical procedures. Nevertheless, there is some evidence that hyperimmunization may lead on occasions to more potent anti-lymphocytic sera capable of suppressing thymus independent responses.

TABLE 39 - A SUMMARY OF THE ABILITY OF A SINGLE ALG PREPARATION (1d) TO SUPPRESS PRIMARY AND SECONDARY HUMORAL RESPONSES IN MICE[a]

| Antigen | Alum BSA[b] (1 mg IP) | | Sheep Erythrocytes $3 \times 10^8$ IP | | Type III Polysaccharide |
|---|---|---|---|---|---|
| Response / Strain | Primary | Secondary | Primary | Secondary | Primary |
| A/HeJ | Yes[c] | No | Yes | Yes | Yes |
| $C_{57}Bl$ | Yes | Variable | Yes | Yes | Yes |
| Balb/c | No | Not Tested | Yes | Yes | Yes |
| DBA/1 | Yes | Not Tested | Yes | Yes | Yes |
| CBA | Yes | No | Yes | Yes | Yes |
| $C_3H$ | Yes | Variable | Yes | Yes | Yes |

(a) The observations summarised were noted with a standard ALG treatment schedule (day -4 and -2 in relation to antigen given on day 0). Alternative schedules can have different effects.

(b) James and Milne 1972 (expect primary anti-BSA response in CBA mice).

(c) Indicates that the suppression achieved was statistically significant ($P<0.05$).

TABLE 40 - A SUMMARY OF THE ABILITY OF VARIOUS ALG PREPARATIONS TO SUPPRESS PRIMARY HUMORAL IMMUNE RESPONSES

| Antigen / ALG Prep.[b] | BSA | SRBC | | SSS-III | |
|---|---|---|---|---|---|
| | | IgM | IgG | -4;-2[d] | 0[d,e] |
| 1a | Yes[c] | No | Yes | No | - |
| 1b | Yes | Yes | Yes | Yes | - |
| 1c | Yes | Yes | Yes | Yes | - |
| 1d | Yes | Yes | Yes | Yes | Yes |
| 2 | Yes | Yes | Yes | Yes)[f] | No |
| 3 | Yes | Yes | Yes | Yes)[f] | No |
| 4 | Yes | Yes | Yes | Yes[g] | No |

(a) All experiments except with SSS-III were carried out in CBA mice only. SSS-III was tested in both CBA and Balb/c mice.

(b) See Table 2 for details.

(c) Indicates that the suppression achieved was statistically significant ($P<0.05$).

(d) Time in days of ALG administration in relation to antigen given on day 0: in case of BSA and SRBC ALG was always administered on day -4 and -2.

(e) Experiments carried out in Balb/c mice.

(f) Suppression was statistically significant only in Balb/c mice but not in CBA mice.

(g) Significant suppression in Balb/c mice whereas a significant potentiation in CBA mice.

It may be relevant to list here certain aspects of the immunosuppressive properties of ALG which should be investigated further. For instance, it would be interesting to test the immunosuppressive properties of several anti-lymphocytic sera produced in different species by different immunization methods (e.g. different immunization schedules; with and without the use of adjuvants, etc.). As the studies reported in this thesis were not designed to explain the mode of action of ALG in humoral immune responses it would be interesting to perform further experiments and to determine the target of ALG action. In this connection experiments should be undertaken to confirm if thymus cells can really restore the capacity of ALG-treated animals to respond to SSS-III.

In view of the recent findings of Jeejeebhoy and Singla (1972a) it would be interesting to ascertain if ALG treatment results in the release of soluble factors which may influence immunological processes. It has been shown in vitro the the binding of ALG to lymphocytes may result in the formation of soluble factors which interfere with the normal behaviour of macrophages (Caspary, Hughes and Field, 1970). This hypothesis may provide additional explanation for the lack of correlation between lymphopenia and immunosuppression following ALG treatment.

The effect of ALG on the production of antibody in different immunoglobulin classes and subclasses undoubtedly

requires further investigation. This is especially so in the case of primary immune responses and should be investigated with a variety of antigens and adjuvants. Furthermore, studies into the adjuvant effects of the ALG treatment should be undertaken. While, it is established that most ALG preparations cause immunosuppression when given in large dose, they could possibly potentiate immune responses when administered at suboptimal doses.

The use of ALG as a carrier for antigens should also be explored. It is conceivable that the accessibility of the antigen (local concentration) around the lymphoid cells may be the limiting factor in determining the immunogenicity of certain antigens and this accessibility could be facilitated by the linkage of the antigen to a non-cytolytic form of ALG ( for example, IgG F(ab)2 ).

As previously stressed these studies were not designed to determine the mode of action of anti-lymphocytic sera. However, it may be relevant to comment here on the possible mechanisms by which these reagents may cause the suppression of cellular and humoral immune responses.

The most likely targets of action of anti-lymphocytic sera are the cells of the lymphoreticular system, the recirculating long-lived antigen sensitive cells of thymic origin being the most popular candidate (Turk and Willoughby, 1967; Taub and Lance, 1968; Denman, Denman and Embling, 1968; Everett, Schwarz, Tyler and Perkins, 1970). The

temporary inactivation or destruction of this cell population by anti-lymphocytic sera is believed to account for the suppression by ALG of all cell-mediated immune responses (Medawar, 1969; Tursi, Greaves, Torrigiani, Playfair and Roitt, 1969; Lance, 1970a) as well as most humoral immune responses (Moller and Zukowski, 1968; Martin and Miller, 1968; Leuchars, Wallis and Davies, 1968). However, there is now sufficient evidence to indicate that anti-lymphocytic sera may interfere with the thymus independent (B) lymphocyte population (DeMeester, Anderson and Shaffer, 1968) which may ultimately result in the suppression of humoral immune responses (Jeejeebhoy, 1970). The suppression of certain humoral immune responses which are independent of thymic cell participation by anti-lymphocytic sera (Shellam, 1969; Allan, Freeman and Mills, 1970; James and Milne, 1971) further support this view. In addition, anti-lymphocytic sera have also been shown to act on and interfere with the function of macrophages. The evidence for the effect of these reagents on and the role of macrophages in immune reactions has already been reviewed in the earlier parts of this thesis.

In summary, anti-lymphocytic sera may exert their effect upon immune responses by interacting with several components of the lymphoreticular system. While in general it would be true to say that these reagents act preferentially on lymphocytes of the recirculating pool;

as Mitchison (1970) said, "It is simply the soldiers who stick their heads above the parapet who get shot."

The exact mechanism of cellular elimination may include complement mediated cytotoxic destruction and phagocytosis of opsonized cells. Alternative but less likely mechanisms of cellular inactivation include blindfolding, enhancement, sterile inactivation, antigen competition, etc. In addition to cellular targets, anti-lymphocytic sera have also been proposed to act via non-cellular factors such as thymic humoral factors (Nagaya, 1970), lymph node permeability factor (Turk and Willoughby, 1969), immunoglobulin receptors (Huber, Michlmayr and Fudenberg, 1969; James, Pullar, James, Wood, Epps and Rahr, 1970), and complement (Willoughby, 1968).

It should be stressed here that although the results recorded in this thesis do not specifically support any of the views on the mode of action of anti-lymphocytic sera (see James, 1973), they do not contrast with any of the theories so far advanced. While the results discussed earlier provide information on how ALG interferes with the function of cells of the lymphoreticular system, they favour the view that this reagent may on occasions interfere with the function of both 'T' and 'B' cells and possibly macrophages. The implications of these cells in the mechanism of humoral immune responses has already been stressed.

# APPENDIX I

## BUFFERS AND SOLUTIONS

PHOSPHATE BUFFER

Solution A

$Na_2HPO_4$ 71.6 gm

Make up to one litre with distilled water

Solution B

$NaH_2PO_4$ 31.2 gm

Make up to one litre with distilled water

0.01M; pH 7.5 buffer

168 ml solution A plus 32 ml solution B; make up to 4 litres with distilled water

0.01M; pH 8.0 buffer

180 ml solution A plus 20 ml solution B; make up to 4 litres with distilled water.

PHOSPHATE BUFFERED SALINE (pH 7.2)

400 ml 1.5M (87.675 gm per litre) NaCl

900 ml phosphate buffer solution A

300 ml phosphate buffer solution B

Mix and make up to 4 litres with distilled water

## HANK'S SOLUTION

### Stock Solution A

1) NaCl 80 gm

KCl 4 gm

$MgSO_4$ ($7\ H_2O$) 1 gm

MgCl ($6\ H_2O$) 1 gm

Dissolve in 400 ml distilled water

2) $CaCl_2$ (anhydrous) 1.4 gm

Dissolved in 50 ml distilled water

Add solutions 2 and 1 slowly with constant mixing and adjust the volume to 500 ml with distilled water.

Sterilize by autoclaving at 10lb. p.s.i. for 10 minutes.

### Stock Solution B

$Na_2HPO_4$ ($12\ H_2O$) 1.52 gm

$KH_2PO_4$ 0.60 gm

Glucose 10.00 gm

Dissolve in distilled water and make up to 500 ml

Sterilize by autoclaving at 10lb. p.s.i. for 10 minutes

### Stock Solution C

$NaHCO_3$ 1.4 gm in 100 ml distilled water. Sterilized by ultrafiltration. Saturate with $CO_2$ and store in 5 ml. aliquots.

To obtain a working Hank's solution, mix 5 ml of solution A, 5 ml of solution B and 2.5 ml of solution C and make up to 100 ml with sterile distilled water.

TRYPAN BLUE

1 gm trypan blue in 100 ml distilled water. Filter and store sterile.

To make a working solution add 1 ml stock solution to 14 ml of Hank's solution.

VERONAL BUFFERED SALINE

| | | |
|---|---|---|
| NaCl | 85.00 | gm |
| $MgCl_2$ (6 $H_2O$) | 16.8 | gm |
| $CaCl_2$ (anhydrous) | 0.28 | gm |
| 5:5-diethyl barbituric acid | 5.575 | gm |
| Na-barbiturate | 2.0 | gm |

Dissolve in distilled water and make up to 200 ml.

Dilute 1 in 5 in distilled water before use.

BORATE BUFFER (pH 8.3-8.5)

| | |
|---|---|
| Boric acid | 6.184 gm |
| Sodium tetraborate | 9.536 gm |
| Sodium chloride | 4.384 gm |

Dissolve in distilled water and make up to 1 litre.

# APPENDIX II

## VARIATIONS IN THE IMMUNE RESPONSE OF DIFFERENT MOUSE STRAINS

In the course of studies on the effect of ALG on the humoral immune responses of different mouse strains to SRBC and SSS-III it appeared from the immune response of control animals that different strains of mice varied significantly in their ability to respond to these antigens. Furthermore, earlier studies carried out in this laboratory on the effect of ALG on the immune response to BSA in different mouse strains indicated similar interstrain variations (James and Milne, 1972).

For a number of reasons, however, it was not possible to draw firm conclusions on these interstrain differences. Firstly, not all the experiments with one particular antigen were carried out simultaneously and therefore it was not possible to account for the variability from one experiment to the other. In addition, the prior treatment with normal horse IgG could have modified the response to test antigens and might have varied from strain to strain. In this connection it is interesting to note that Barth and his colleagues reported a suppression in the immune response of Balb/c mice against SRBC by prior treatment with normal rabbit IgG (Barth and Southworth, 1968; Barth, Hunter and Southworth, 1969; Barth and Carroll, 1970). The same group of workers also reported the suppression of anti-SSS-III response of Balb/c mice by normal burro serum treatment (Baker, Barth, Stashak and Amsbaugh, 1970; Barth, Baker, Stashak and Amsbaugh, 1971).

A similar suppression of anti-SRBC response by normal rabbit IgG has been reported in both rats (Baum, Lieberman and Frenkel, 1969), and mice (Baroni, Kimbal, Ward and Wagar, 1969). In contrast, Anderson and her colleagues observed an enhancement of anti-SRBC response by normal rabbit globulin (Anderson, Dresser, Iverson, Lance, Wortis and Zebra, 1972). Similarly, moderate to severe enhancement of antibody responses by the normal rabbit globulin treatment have been recorded by various other workers (Marshall and Knight, 1969; Baroni, Kimbal, Ward and Wagar, 1969; Jeejeebhoy and Singla, 1972a).

It was therefore felt necessary to study the immune response of different mouse strains to SRBC, SSS-III and BSA under experimental conditions that would permit a meaningful interstrain comparison.

Mice of strains listed in Table 1 were immunized with a suboptimal dose of SRBC ($3 \times 10^7$), SSS-III (0.1 µg.) and BSA (0.1 mg.). In certain experiments mice were also immunized with 1.0 mg. BSA. In all experiments only male mice of comparable age were used. The mode of antigen administration and the assay procedures for measuring immune responses against different antigens were identical with those described in detail in the early parts of this thesis.

The results recorded in Tables 41-43 clearly exhibit an interstrain variation in the response of mice tested to all three antigens. From the results of the total splenic

TABLE 41 - THE IMMUNE RESPONSE OF DIFFERENT MOUSE STRAINS TO SHEEP ERYTHROCYTES (SRBC)[a]

| Exp. No. / Mouse Strain | Direct (IgM) Plaque Forming Cells Per Spleen[b] | | | |
|---|---|---|---|---|
| | 1 | 2 | 3 | 4 |
| A/HeJ | | 14,329(13,010-15,783) (4)[c] | 16,387(11,380-23,594) (4) | |
| $C_{57}Bl$ | 3,959(2,369-6,615) (4) | | | 7,694(4,099-14,444) (5) |
| Balb/c | 32,617(29,820-35,677) (4) | | 38,945(26,575-57,073) (4) | |
| DBA/1 | | | 33,752(31,669-35,972) (4) | 17,561(16,124-19,125) (5) |
| CBA | | 25,591(22,562-29,028) (5) | 45,907(36,725-57,385) (4) | 19,806(18,089-21,687) (4) |
| $C_3H$ | 6,515(4,919-8,628) (5) | | | 11,802(7,880-17,674) (5) |

(a) $3 \times 10^7$ SRBC injected i.p. on day 0 and tested on day 5.

(b) Geometric mean with the limits of one standard error.

(c) Number of mice per group.

TABLE 42 - THE IMMUNE RESPONSE OF DIFFERENT MOUSE STRAINS TO PNEUMOCOCCAL POLYSACCHARIDE (SSS-III)[a]

| Mouse Strain \ Exp. No. | Plaque Forming Cells Per Spleen[b] | | | |
|---|---|---|---|---|
| | 1 | 2 | 3 | 4 |
| A/HeJ | | 1,777(1,398-2,258) (4)[c] | 980(727-1,321) (4) | 1,483(1,206-1,823) (4) |
| $C_{57}Bl$ | | | 213(134-338) (4) | 1,954(1,791-2,131) (5) |
| Balb/c | 7,515(5,958-9,479) (5) | | | 15,939(13,456-18,880) (5) |
| DBA/1 | | | | 1,908(1,466-2,484) (5) |
| CBA | | 4,625(3,183-6,718) (4) | | 2,932(2,191-3,924) (5) |
| $C_3H$ | | 4,710(3,717-5,967) (5) | 5,736(4,077-8,071) (4) | |

(a) 0.1 µg. SSS-III injected i.v. on day 0 and tested on day 5.

(b) Geometric mean with the limits of one standard error.

(c) Number of mice per group.

TABLE 43 - IMMUNE RESPONSE OF DIFFERENT MOUSE STRAINS TO BOVINE SERUM ALBUMIN (BSA)[a]

| Mouse Strain | 0.1 mg. BSA | | 1.0 mg. BSA | |
|---|---|---|---|---|
| | Antigen Binding Capacity[b] | Relative Binding Affinity | Antigen Binding Capacity | Relative Binding Affinity[c] |
| A/HeJ | 4.842 (4.182-5.606) (5)[d] | N.T. | 8.122 (7.434-8.873) (5) | 37.1 ± 1.1 (5) |
| $C_{57}Bl$ | 0.116 (0.085-0.156) (6) | N.T. | 0.910 (0.754-1.098) (6) | 34.3 ± 3.9 (6) |
| Balb/c | 0.853 (0.702-1.037) (5) | N.T. | 2.037 (1.928-2.153) (6) | 28.3 ± 2.7 (6) |
| DBA/1 | N.T. | N.T. | 3.086 (2.447-3.890) (5) | 44.8 ± 7.2 (5) |
| CBA | 2.551 (1.953-3.333) (5) | N.T. | 2.947 (2.337-3.715) (6) | 34.1 ± 2.4 (6) |
| $C_3H$ | 1.791 (1.559-2.057) (6) | N.T. | 3.117 (2.198-4.419) (6) | 52.4 ± 5.9 (6) |

(a) 0.1 or 1.0 mg. BSA (alum precipitated) injected i.p. on day 0 and tested on day 20.

(b) Geometric mean with the limits of one standard error.

(c) Arithmetic mean ± 1 standard error.

(d) Number of mice per group.

PFC response against SRBC it is apparent that $C_{57}Bl$, A/HeJ and $C_3H$ mice respond poorly to this antigen, whereas Balb/c, CBA and DBA/1 mice give relatively high responses (Table 41). This is also illustrated by the number of PFC responses per $10^6$ nucleated spleen cells in different strains summarized in Figure 23. Incidentally, the closed circles in Figures 23-26 represent the responses of individual mice.

The strain variations in the immune response to SSS-III followed a pattern somewhat similar to that of anti-SRBC response with the exception of DBA/1 and $C_3H$ mice. It is apparent that A/HeJ, $C_{57}Bl$ and DBA/1 mice always responded poorly whereas Balb/c mice responded favourably to this antigen. It should be noted that $C_3H$ mice which responded poorly compared with CBA mice when tested against SRBC gave an anti-SSS-III response which was comparable to that in CBA mice. It should also be noted that DBA/1 mice which gave a favourable anti-SRBC response failed to give an anti-SSS-III response comparable to that observed in Balb/c mice (Figure 24).

The pattern of interstrain variations in the responsiveness of mice tested against BSA was grosslydifferent from that obtained with SRBC or SSS-III. However it is interesting to note that $C_{57}Bl$ mice still failed to respond favourably to this antigen (Table 43 and Figures 25 and 26). A/HeJ mice which consistently responded poorly to the other two

FIGURE 23

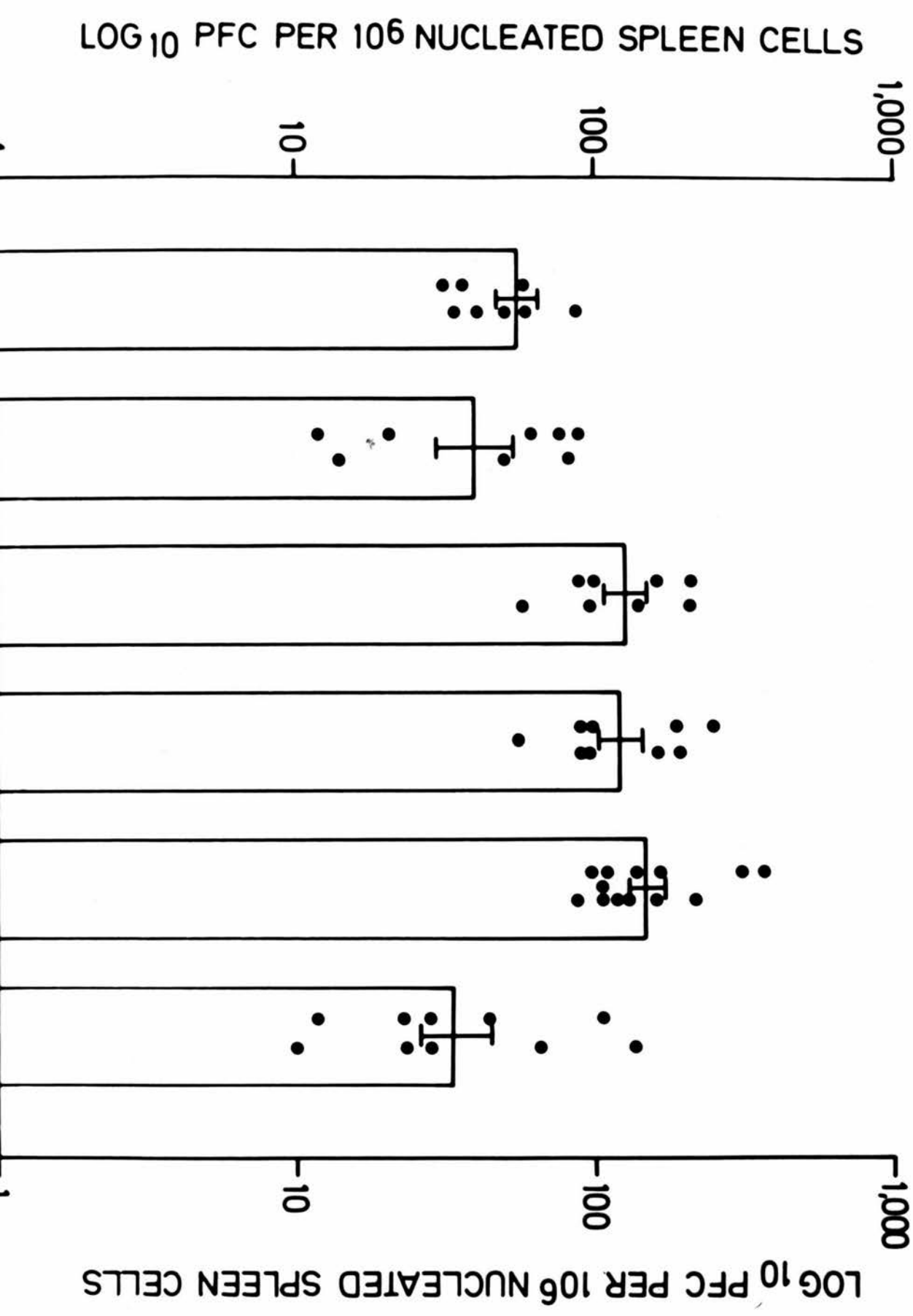

LOG 10 PFC PER 10⁶ NUCLEATED SPLEEN CELLS
1,000
100
10
1
PRIMARY IMMUNE RESPONSE OF DIFFERENT MOUSE STRAINS TO SRBC
LOG 10 PFC PER 10⁶ NUCLEATED SPLEEN CELLS
1,000
100
10
1

FIGURE 24

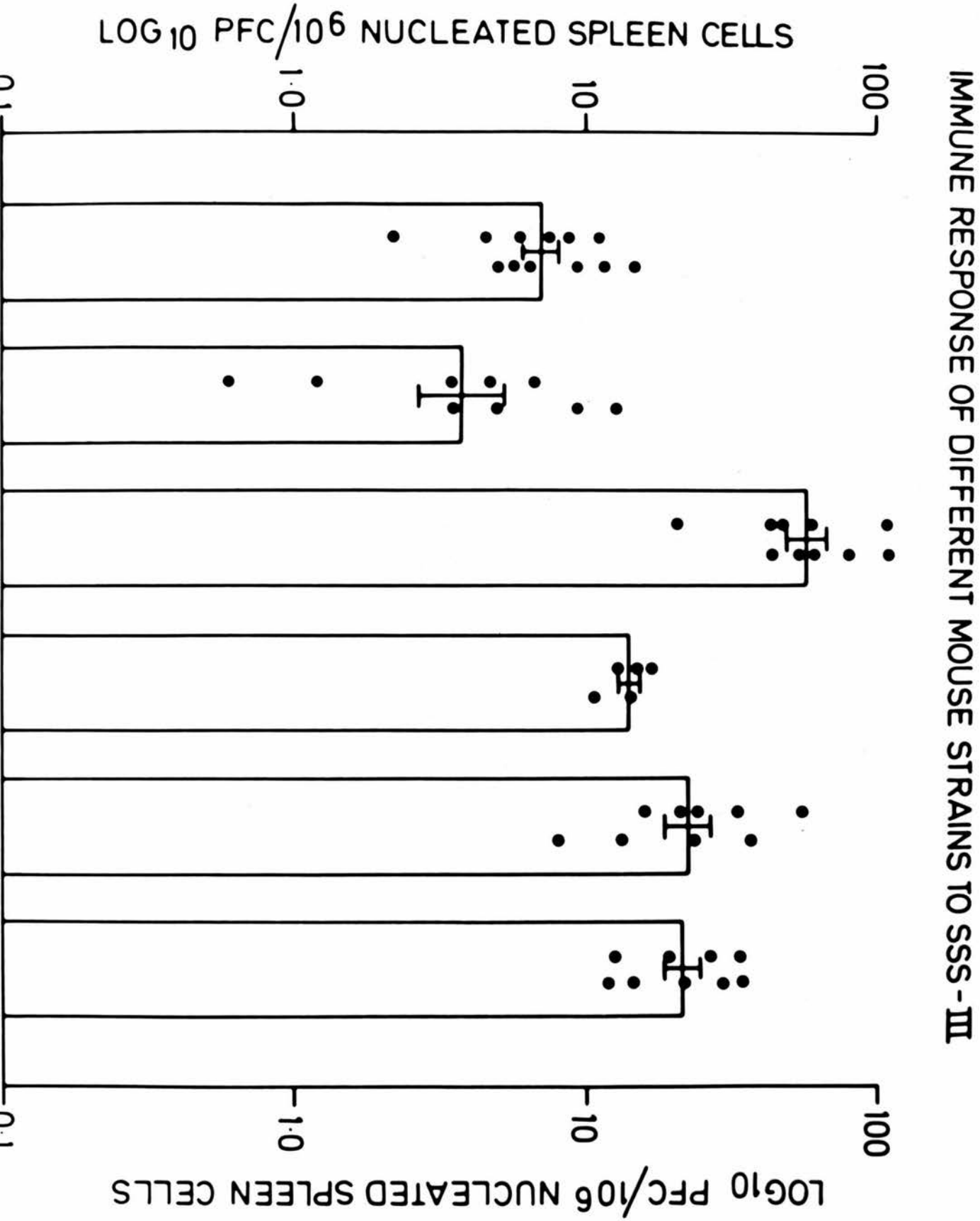
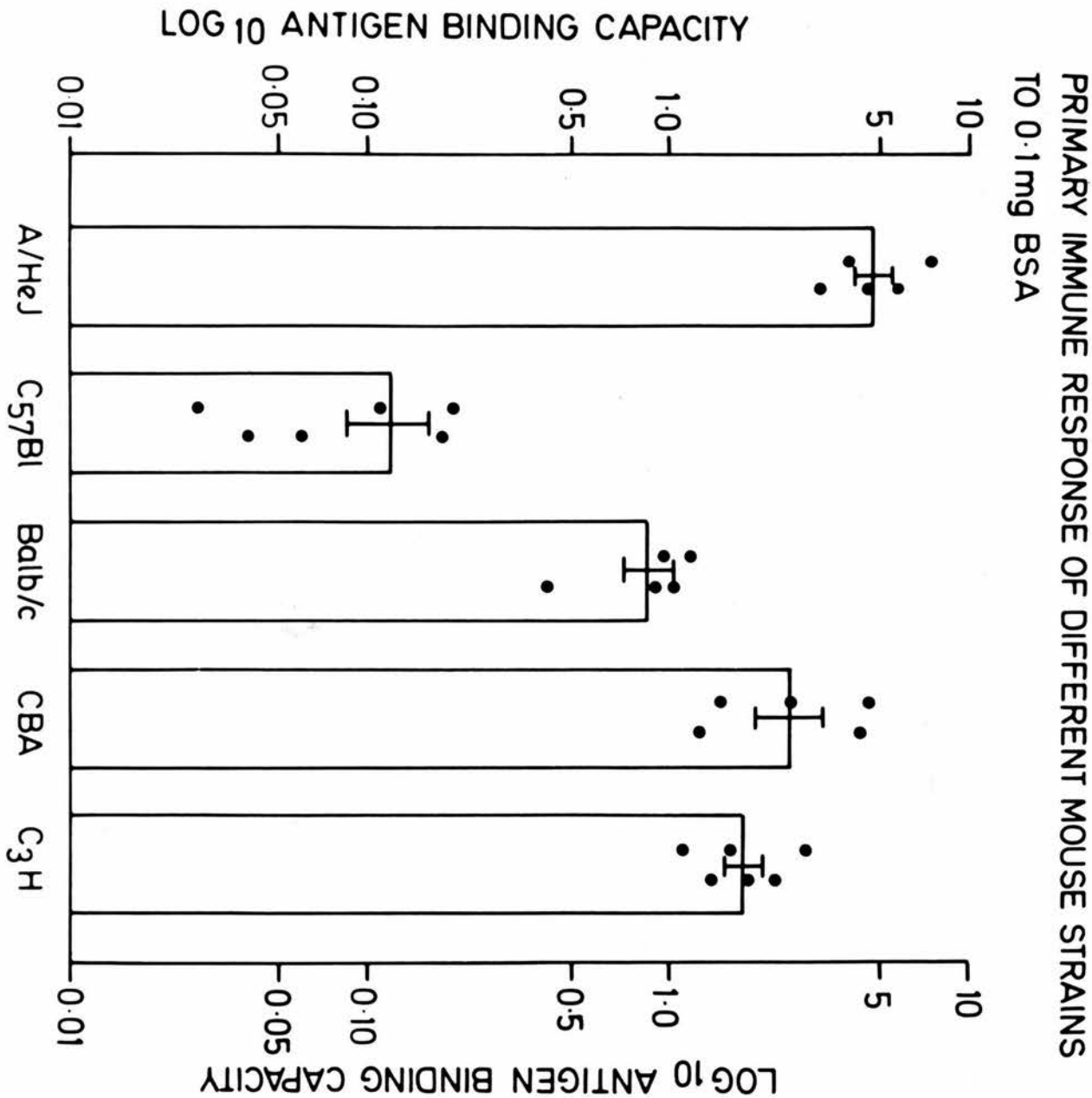

LOG 10 PFC/10⁶ NUCLEATED SPLEEN CELLS
0·1
1·0
10
100
IMMUNE RESPONSE OF DIFFERENT MOUSE STRAINS TO SSS-III
LOG10 PFC/10⁶ NUCLEATED SPLEEN CELLS
0·1
1·0
10
100

FIGURE 25


PRIMARY IMMUNE RESPONSE OF DIFFERENT MOUSE STRAINS
TO 0·1 mg BSA
LOG 10 ANTIGEN BINDING CAPACITY
0·01
0·05
0·10
0·5
1·0
5
10
A/HeJ
C57Bl
Balb/c
CBA
C3H

antigens appeared to be the best responders to BSA, whereas Balb/c mice which were the best responders against SRBC and SSS-III gave only a poor to moderate response against BSA. It should be noted that the interstrain variations in the responsiveness to this antigen were more distinct in mice challenged with the lower antigen dose (0.1 mg.; Figure 26). It is apparent that when 1 mg. BSA was administered Balb/c, DBA/1, CBA and $C_3H$ mice gave comparable responses which were between the extreme responses given by A/HeJ and $C_{57}Bl$ mice (Figure 26).

The results of relative binding affinity determinations did not in any way correlate with the responsiveness of different mouse strains (Figure 27). However, striking differences were noticed in the relative binding affinities of anti-BSA antibodies produced by different strains.

Quantitative differences in the ability of different mouse strains to respond to SRBC (Stern, Brown and Davidsohn, 1956; Playfair, 1968; Buschman, Krausslich, Meyer, Radzikowski and Osterkorn, 1972), bacterial polysaccharides (Braley and Freeman, 1971; DiPauli, 1972; Braun, Kindred and Jacobson, 1972) and other protein, polysaccharide and poly-amino acid antigens (McDevitt and Chinitz, 1969; McDevitt and Benacerraf, 1969; Cerottini, Lambert and Dixon, 1969; Vaz, Vaz and Levine, 1970; Lieberman and Humphrey, 1972) have been reported. The findings documented here generally confirm the findings of previous workers. The hypo-

FIGURE 26

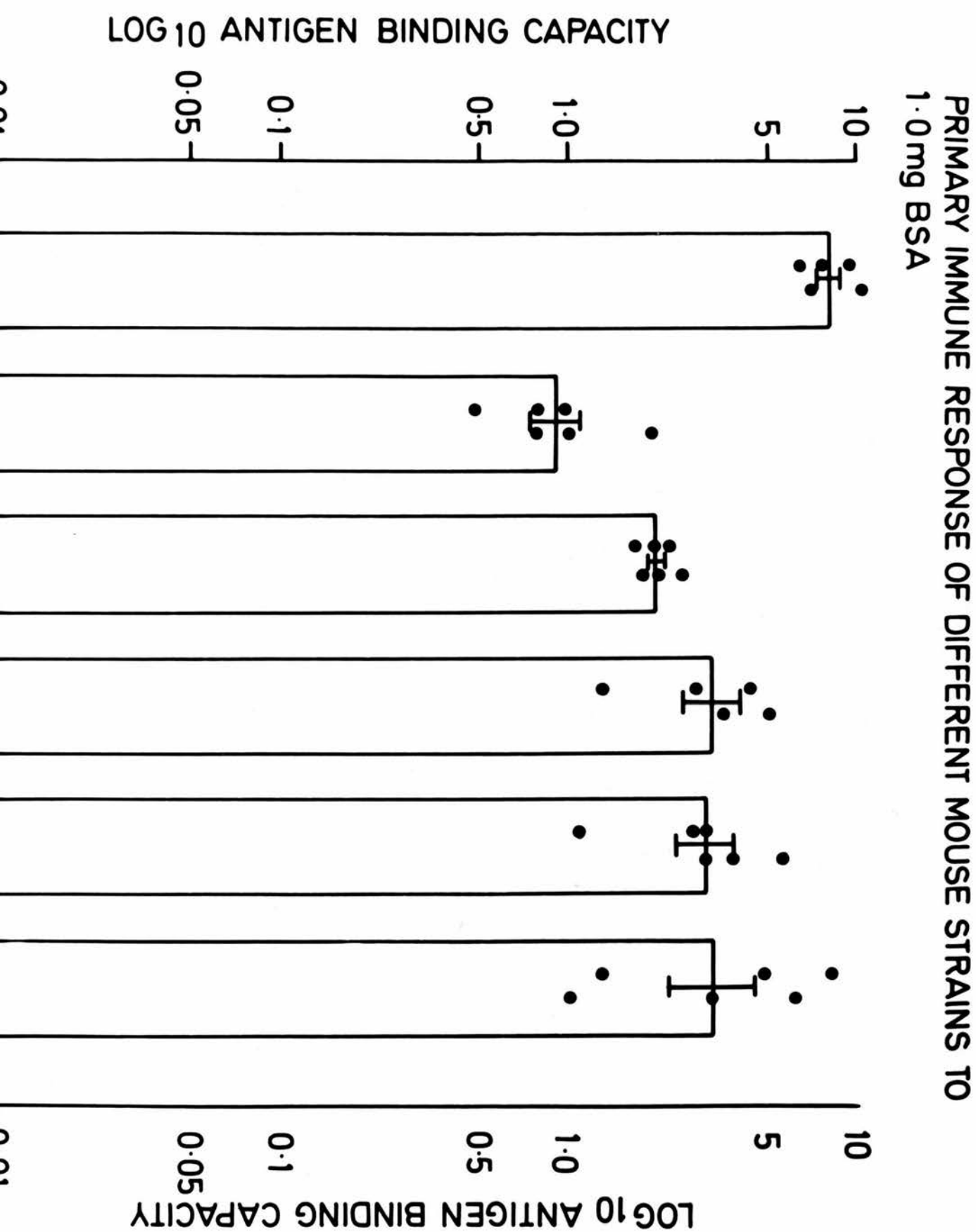
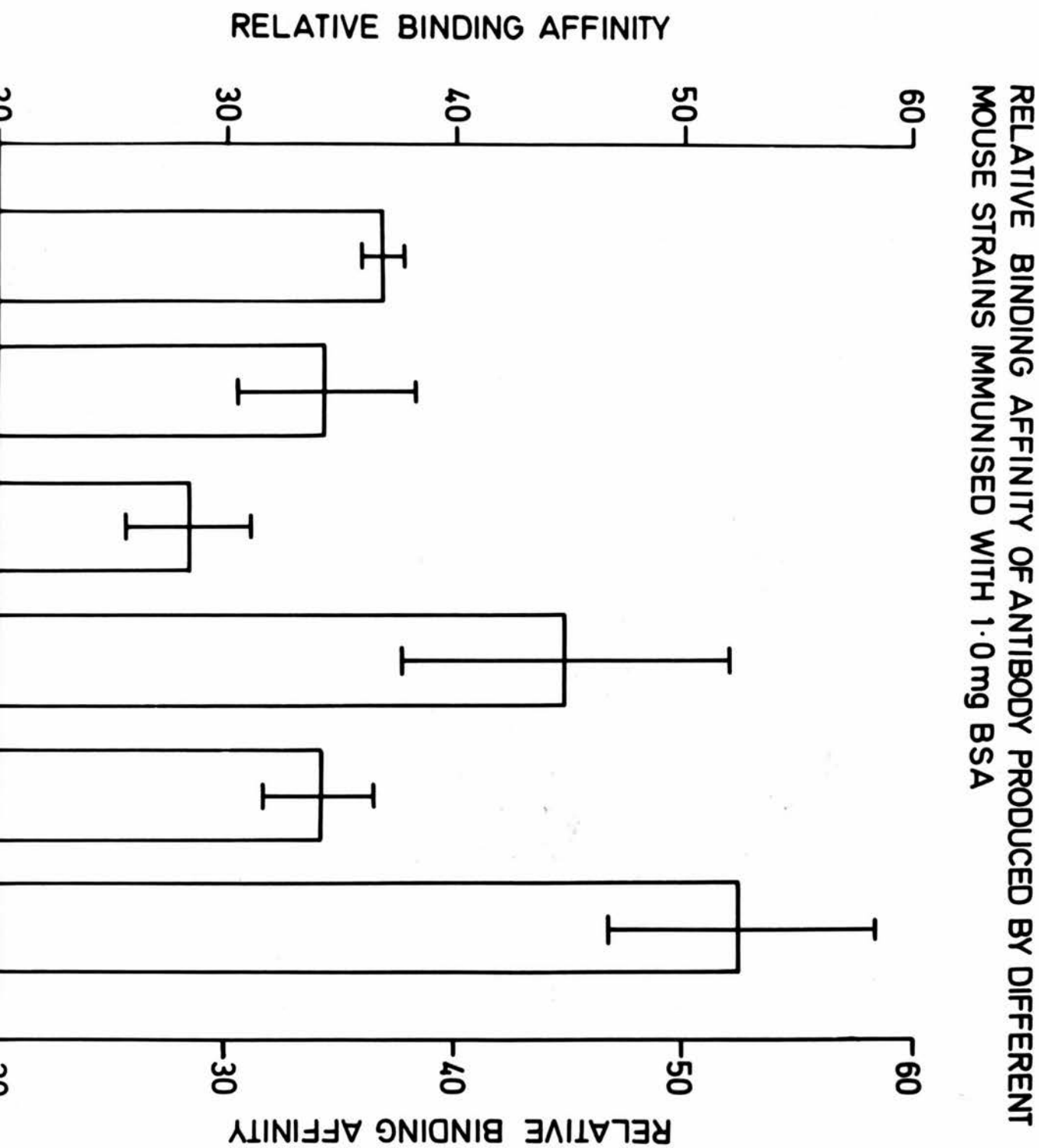

LOG 10 ANTIGEN BINDING CAPACITY
0·05
0·1
0·5
1·0
5
10
PRIMARY IMMUNE RESPONSE OF DIFFERENT MOUSE STRAINS TO 1·0 mg BSA
LOG 10 ANTIGEN BINDING CAPACITY
0·05
0·1
0·5
1·0
5
10

FIGURE 27


RELATIVE BINDING AFFINITY
30
40
50
60
RELATIVE BINDING AFFINITY
RELATIVE BINDING AFFINITY OF ANTIBODY PRODUCED BY DIFFERENT MOUSE STRAINS IMMUNISED WITH 1·0 mg BSA

responsiveness of $C_{57}Bl$ mice and the hyper-responsiveness of Balb/c mice to SRBC is in general agreement with the findings of Playfair (1968).

Similarly the variations in the responsiveness of different mice against SSS-III are consistent with the findings previously reported by Braley and Freeman (1972). Thus, Balb/c mice gave the highest response to this antigen, whereas the response of $C_{57}Bl$ and A/HeJ mice was extremely low. However, in contrast to their findings indicating a difference in the responsiveness of CBA and $C_3H$ mice (the former being the poorer of the two) the results reported here indicated a comparable response in the two mouse strains (Figure 24).

The variations in the responsiveness of different strains is more striking in experiments with BSA (Figure 25) although these differences are relatively less marked in mice challenged with the higher antigen dose (Figure 26). These results are in agreement with those of Cerottini, Lambert and Dixon (1969) in as much as the hyper-responsiveness of A/HeJ mice is concerned. However in contrast with the findings recorded here Cerottini and his colleagues found that CBA mice responded poorly in comparison with Balb/c mice. This difference may however be due to the modification of the immune response of one or both the mouse strains by the use of Freund's complete adjuvant by these authors (Cerottini, Lambert and Dixon, 1969).

In addition, the affinities of anti-BSA antibody produced varied from strain to strain (Figure 27). It is interesting to note that similar interstrain variations in the affinity of anti-BSA antibody have been previously reported (Soothill and Steward, 1971; Petty, Steward and Soothill, 1972). However, these results contrast with those of Cerottini and his colleagues who failed to notice any interstrain variations in the relative binding affinity of anti-BSA antibody produced by different mouse strains (Cerottini, Lambert and Dixon, 1969). This could perhaps again be due to the use of Freund's complete adjuvant in their experiments. It should be noted that there was no correlation between the responsiveness of different mouse strains and the affinity of antibody produced. Thus, CBA and $C_3H$ mice which responded comparably to BSA did not produce antibodies of the same relative binding affinity (see Figure 27).

In several previous reports it has been shown that strains of mice which are hypo-responsive to one antigen, may exhibit normal or enhanced response to other antigens. Thus, Playfair (1968) observed that although NZB mice responded favourably to SRBC compared with Balb/c mice, the two strains gave comparable responses against pig and chicken erythrocytes. Nevertheless, he too found that $C_{57}Bl$ mice were unable to respond favourably to any of the antigens he tested. Similarly, McDevitt and Chinitz

(1969) showed that mice which responded poorly to (H,G)-A-L gave a good response to (T,G)-A-L and (Phe,G)-A-L and those which responded well to (Phe,G)-A-L gave only a meagre response to (T,G)-A-L and (H,G)-A-L. This pattern in the responsiveness of different mouse strains to various antigens has been noted by others (Cerottini, Lambert and Dixon, 1969).

The findings reported here generally confirm the observations referred to above. For example, A/HeJ mice which produced the best response against BSA gave very poor responses against SRBC and SSS-III. Conversely, Balb/c mice which gave the highest responses against SRBC and SSS-III produced only a modest response against BSA. In addition DBA/1 mice which were as good as Balb/c mice in their anti-SRBC response did not give an anti-SSS-III response comparable with Balb/c mice. It is however interesting to note that $C_{57}$Bl mice remained throughout the poorest responders to all three antigens. It should be recalled that this mouse strain has been reported to give a very poor response to several other antigens (Playfair, 1968; Braun, Kindred and Jacobsen, 1972).

Finally, a relationship between histocompatibility (H-2) genotype and the responsiveness to different antigens has been repeatedly reported (McDevitt and Benaceraff, 1969; Skarova and Riha, 1969; Gasser, 1969; Tyan, McDevitt and Herzenberg, 1969; McDevitt and Chinitz, 1969; Vaz, Vaz

and Levine, 1970). However, from the studies reported here it is not possible to conclude whether or not the responsiveness to different antigens is related to the H-2 genotype, although there is some suggestion that it may be so. This is reflected by the comparable responsiveness of $C_3H$ and CBA (both H-$2^k$) mice to SSS-III and BSA. In contrast, the response of these two strains did not appear to be comparable when challenged with SRBC. This may be due to the complex nature of the latter antigen. It should, however, again be stressed that the data from experiments reported here are not adequate enough to draw any conclusions on the genetic basis of the immune responses.

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